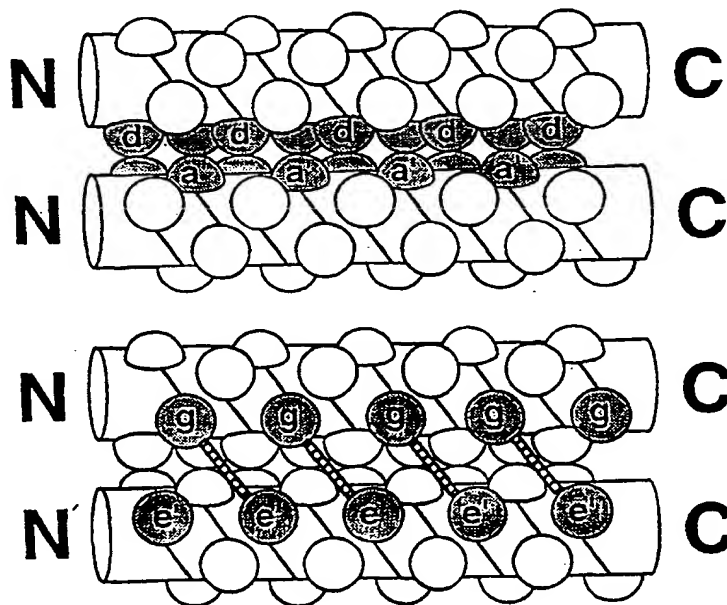




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/11, C07K 14/00, 14/47, 14/025, 14/39, 14/435, 14/16, G01N 33/68		A1	(11) International Publication Number: WO 98/32848 (43) International Publication Date: 30 July 1998 (30.07.98)
(21) International Application Number: PCT/US98/01871 (22) International Filing Date: 28 January 1998 (28.01.98) (30) Priority Data: 60/036,219 28 January 1997 (28.01.97) US (71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; Suite 510, 2150 Shattuck Avenue, Berkeley, CA 94720 (US). (72) Inventors: ALBER, Thomas, C.; 954 San Benito Road, Berkeley, CA 94707 (US). ALLEN, Victoria; 2729 Piedmont Avenue #302, Berkeley, CA 94704 (US). NAUTYAL, Shivani; 1350 Euclid Avenue, Berkeley, CA 94704 (US). (74) Agents: TRECARTIN, Richard, F. et al.; Flehr, Hohbach, Test, Albritton & Herbert LLP, Suite 3400, 4 Embarcadero Center, San Francisco, CA 94111-4187 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	

(54) Title: PEPTIDE PROBES TO COIL PROTEINS AND METHODS FOR MAKING THE SAME



(57) Abstract

The object of the present application is to provide heterospecific peptide structures directed to coiled-coil regions of determined proteins and method to manufacture and use these peptides.

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PEPTIDE PROBES TO COIL PROTEINS AND METHODS FOR MAKING THE SAME

FIELD OF THE INVENTION

The present invention relates generally to the field of biological probes, and more specifically to polypeptide probes directed to coiled coil proteins.

5 This invention was made with Government support under Grant No. RO1GM48958 awarded by the National Institutes of Health. The Government has certain rights to this invention.

BACKGROUND OF THE INVENTION

10 Interactions among proteins are essential for the controlled function of all living cells, and inappropriate or adventitious protein-protein interactions are the hallmarks of virtually all diseases. Accordingly, biological probes directed at either the proteins themselves, or alternatively at their nucleic acid precursors, are essential for diagnosis of disease and general human health.

Existing biological probes are either nucleic acid-based or amino acid-based. 15 Unfortunately, these prior art biological probes suffer from distinct and somewhat complementary disadvantages. Nucleic-acid based systems include oligonucleotides targeted for specific RNA or DNA sequences, which have the advantages of straightforward synthesis and simple rules for molecular complementarity. However, at present they can only be targeted easily to nucleic acids. Amino acid-based 20 biological probes include antibodies, which can recognize a much broader range of molecules than nucleic acid-based probes. However, they are expensive and time-consuming to produce, requiring purification of the target and maintenance of

animals. Moreover, functional antibodies cannot be expressed in intracellular compartments, and their use as biological probes often suffers from varying degrees of antigen expression, non-specific binding and adverse immunogenic reactions.

Accordingly, there is a significant need in the art for a biological probe with broader applications than oligonucleotides and antibodies. Such a probe should combine the advantages of nucleic acid-based probes – quick synthesis, intracellular expression and easy labeling *in vitro* – with the specificity of antibodies for peptide sequences. Ideally, these probes should be sensitive, stable and recognize a wide range of biological molecules having different biological functions.

10 SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to remedy the disadvantages encountered in prior art biological probes, through the provision of heterospecific polypeptide probes directed to coiled coil regions, and novel methods for making and using these probes.

15 In one embodiment, the present invention contemplates a polypeptide probe having a coiled coil region, wherein the core amino acid residues of the probe coiled coil region have been selected according to the core residue pairing rules so as to favor heterospecific oligomerization with a target coiled coil region. In a further embodiment, the amino acid residues of the probe coiled coil region are selected so as
20 to optimize electrostatic interactions in the edge region of the interhelical interface adjacent to the core. In a specific embodiment, the probe comprises a substantially purified polypeptide sequence (SEQ ID NO:) directed to the APC protein. In a further embodiment, the invention comprises an isolated polynucleotide sequence encoding the polypeptide of SEQ ID NO: .

25 In an alternative embodiment, the present invention comprises a coiled coil structure formed between a target coiled coil region and a probe coiled coil region, wherein the amino acid residues located at positions a' and d' in the heptads of the probe coiled coil region are selected according to the core residue pairing rules. In a preferred embodiment, a leucine located at position a in a heptad of the target coiled
30 coil region is paired with a preferred amino acid in the a' position in a heptad of the

probe coiled coil region, wherein the preferred amino acid is selected from the group comprising: phenylalanine, isoleucine, lysine, methionine, serine, threonine and valine. In a further embodiment, a leucine located at position **d** in a heptad of the target coiled coil region is paired with a preferred amino acid in the **d'** position in a heptad of the probe coiled coil region, wherein said preferred amino acid is selected from the group comprising tryptophan, valine, arginine, glutamine, phenylalanine, asparagine, methionine, isoleucine and alanine.

In another preferred embodiment, a valine located at position **a** in a heptad of the target coiled coil region is paired with a preferred amino acid in the **a'** position in a heptad of the probe coiled coil region, wherein the preferred amino acid is selected from the group comprising: alanine, isoleucine, leucine, glutamine and serine. In a further embodiment, a valine located at position **d** in a heptad of the target coiled coil region is paired with a preferred amino acid in the **d'** position in a heptad of the probe coiled coil region, wherein said preferred amino acid is selected from the group comprising tyrosine, alanine, leucine, methionine, glutamine and threonine.

In an alternative embodiment, the present invention provides a hetero-oligomer comprising a polypeptide probe and a polypeptide target, wherein the probe comprises a first core region and the target comprises a second core region, and wherein a leucine in an **a** position in the second core region is paired with a preferred amino acid in an **a'** position of the first core region, wherein the preferred amino acid in the **a'** position is selected from the group comprising phenylalanine, isoleucine, lysine, methionine, serine, threonine, and valine. In a further embodiment, a leucine in the **d** position of the second core region is paired with a preferred amino acid in the **d'** position of the first core region, wherein the preferred amino acid in the **d'** position is selected from the group comprising tryptophan, valine, arginine, glutamine, phenylalanine, asparagine, methionine, isoleucine and alanine.

In an alternative embodiment, the present invention provides a hetero-oligomer comprising a polypeptide probe and a polypeptide target, wherein the probe comprises a first core region and the target comprises a second core region, and wherein a valine in an **a** position in the second core region is paired with a preferred amino acid in an **a'** position of the first core region, wherein the preferred amino acid

in the **a'** position is selected from the group comprising alanine, isoleucine, leucine, glutamine and serine. In a further embodiment, a valine in the **d** position of the second core region is paired with a preferred amino acid in the **d'** position of the first core region, wherein the preferred amino acid in the **d'** position is selected from the group comprising tyrosine, alanine, leucine, methionine, glutamine and threonine.

The present invention also provides a heterospecific polypeptide probe directed to a coiled coil region of a target polypeptide, wherein the melting temperature of the hetero-oligomer formed between the probe and the target at a fixed total protein concentration is higher than the melting temperature of a homo-oligomer formed by either the probe or the target. In a preferred embodiment, the melting temperature of the hetero-oligomer is at least 8°C higher than the average melting temperature of the homo-oligomers. In an alternative embodiment, the dissociation constant (K_d) of the hetero-oligomer formed between the probe and the target is lower than the dissociation constant of a homo-oligomer formed by either the probe or the target. In a particularly preferred embodiment, the dissociation constant of the hetero-oligomer is equal to or lower than 10 nM.

One embodiment of the methods of the present invention provides a method for making a heterospecific polypeptide probe, comprising the steps of 1) providing a target polypeptide having a target coiled coil region; 2) determining the target polypeptide sequence of the target coiled coil region; and 3) generating a probe polypeptide having a probe coiled coil region, wherein the probe polypeptide sequence of the probe coiled coil region is selected so as to favor heterospecific oligomerization of the probe coiled coil region to the target coiled coil region. In a preferred embodiment, the probe polypeptide sequence is selected so as to optimize core residue pairing in the interhelical interface between the target coiled coil region and the probe coiled coil region. In a particularly preferred embodiment, the invention contemplates the application of novel core residue pairing rules so as to favor heterospecific pairing between the probe coiled coil region and the target coiled coil region. In a further embodiment, the probe polypeptide sequence is also selected so as to optimize electrostatic interactions in the edge region of the interhelical interface. In a specific embodiment, the target polypeptide sequence is APC polypeptide or a

portion thereof, and the probe polypeptide sequence is the polypeptide of SEQ ID NO: or a portion thereof.

In an alternative embodiment, the invention provides a method for making a heterospecific polypeptide probe, comprising the steps of 1) providing a target
5 polypeptide having at least one target coiled coil region, wherein the target coiled coil region comprises a first core region, a first edge region, and a first exposed region; 2) determining the amino acid sequence of the target coiled coil region; 3) generating a probe polypeptide having a probe coiled coil region, wherein the probe coiled coil
10 region comprises a second core region, a second edge region, and a second exposed region; and 4) selecting amino acids in the second core region so as to optimize the formation of a heterospecific oligomer between the probe polypeptide and the target polypeptide. In a preferred embodiment, the method further comprises the additional step of selecting charged amino acids for the second edge region, wherein the charge
15 on the charged amino acids in the second edge region is the same and is different from the charge on amino acids in the first edge region. In a particularly preferred embodiment, the method also comprises the additional step of selecting amino acids for the second exposed region so as to stabilize the probe coiled coil region.

Another alternative embodiment provides a method for making a heterospecific probe polypeptide sequence, comprising the steps of 1) providing a
20 target polypeptide sequence having a first coiled coil region, wherein the first coiled coil region comprises at least three heptad sequences, with the heptad sequences containing amino acids at positions A, B, C, D, E, F, and G; 2) identifying the amino acids at positions A and D of the first coiled coil region; 3) generating a probe polypeptide sequence having a second coiled coil region, wherein the second coiled
25 coil region comprises at least three heptad sequences, with the heptad sequences containing amino acids at positions A', B', C', D', E', F', and G'; and 4) selecting the amino acids at positions A' and D' such that the probe polypeptide sequence favors hetero-oligomerization with the target polypeptide sequence. In a further embodiment, the method comprises the additional steps of 5) identifying the amino
30 acids at the positions E and G; and 6) selecting the amino acids at the positions E' and G' such that the probe polypeptide favors hetero-oligomerization with the target

polypeptide sequence. In a specific embodiment, the amino acids at positions A' and D' are hydrophobic, and the amino acids at positions E' and G' are charged amino acids. In a still further embodiment, the charge on the amino acids at the positions E' and G' is the same and is different from the charge on the amino acids at the positions E and G.

The present invention also contemplates a method for detecting a coiled coil protein, comprising the steps of 1) providing: a) a target polypeptide sequence having a first coiled coil region; and b) a probe polypeptide sequence having a second coiled coil region; 2) combining the target polypeptide sequence with the probe polypeptide sequence such that a hetero-oligomer is preferentially formed between the target polypeptide sequence and the probe polypeptide sequence; and 3) detecting the hetero-oligomer. In an alternative embodiment, the present invention provides a method for detecting a target polypeptide sequence having a first coiled coil region in a sample, comprising the steps of: 1) providing: a) a sample suspected of containing a first polypeptide sequence; b) a second polypeptide sequence having a second coiled coil region; 2) combining the first polypeptide sequence with the second polypeptide sequence such that a hetero-oligomer is formed between the first polypeptide sequence and the second polypeptide sequence; and 3) detecting the hetero-oligomer.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the core (a and d) and edge (e-to-g) interactions in coiled coils.

Figure 2 shows a helical wheel diagram of the APC-55 peptide and the designed anti-APCp2 polypeptide probe.

Figure 3 shows the circular dichroism spectra of APC and anti-APC polypeptides.

Figure 4 shows the thermal denaturation of APC-55, anti-APCp2, and an equimolar mixture of APC-55 and anti-APCp2.

Figure 5 shows native gel electrophoresis of APC-55, anti-APCp1 and a mixture of APC-55 and anti-APCp1.

Figure 6 shows blots of gels of expressed APC fusion proteins probed with anti-APC monoclonal antibody (A), anti-APCp1 polypeptide (B), and the Comassie Brilliant Blue stained gels used to prepare the blots (C).

Figure 7 shows a schematic diagram of precipitation of wild type and mutant APC from human cell lines using biotinylated anti-APCp2 or biotinylated anti-APC monoclonal antibody.

Figure 8 shows Western blots of wild type and mutant APC from human colon cancer cell lines precipitated using biotinylated anti-APCp2 or biotinylated anti-APC monoclonal antibody.

Figure 9 shows Western blots of precipitations of APC from human colon cancer cell lines 293 (A), DLD-1 (B), and HT-29 (C).

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

The term "heptad" refers to a seven amino acid sequence in which the seven amino acids occupy the positions **a**, **b**, **c**, **d**, **e**, **f**, and **g**. In a given heptad, the amino acid at position **a** is located at the amino-terminal end, whereas the amino acid at position **g** is located at the carboxy-terminal end of the heptad.

The term "coiled coil region" refers to a polypeptide sequence containing two or more repeated heptads, *i.e.*, $(\mathbf{a\ b\ c\ d\ e\ f\ g})_n$, where the **a** and **d** positions are generally, but not exclusively, occupied by hydrophobic residues; the **b**, **c**, **e**, **f** and **g** positions are generally, but not exclusively, occupied by hydrophilic residues; and *n* is a number equal to or greater than 2. A first "coiled coil region" which is placed in apposition with a second coiled coil region, with which it is capable of forming non-covalent bonds, generally adopts a secondary α -helical structure referred to as a "coil structure," as well as a tertiary left-handed superhelical structure referred to interchangeably as a "coiled coil structure" and a "supercoil structure." *See, e.g.*, Lupas, "TIBS 21:375-82 (1996). The distance that the superhelix requires to complete a full turn is called the "pitch," and the angle that each helix assumes relative to the superhelix axis is called the "superhelix crossing angle." The angle

between two neighboring helices is called the "helix-crossing angle." There can be two or more (*e.g.*, three, four or five) helices wound together in the superhelix and they can run either in the same ("parallel") or in opposite ("antiparallel") directions.

5 The term "coiled coil polypeptide" refers to a coiled coil structure comprising two or more polypeptides which in turn comprise one or more coiled coil regions. The polypeptides which comprise a coiled coil polypeptide may have the same or different amino acid sequences. Coiled coil polypeptides containing polypeptides of the same amino acid sequence generally have distinct coiled coil regions with
10 intervening helix-breaking amino acid residues, which allow for protein folding and the oligomerization of succeeding coiled coil regions. For example, the seryl-tRNA-synthetase enzyme comprises an anti-parallel, dimeric coiled coil polypeptide with the two helices joined by a tight turn.

The "interhelical interface" denotes the region on each respective coiled coil region which non-covalently interacts with a neighboring coiled coil region to form a
15 coiled coil. As is known in the art, the interhelical interface comprises the "hydrophobic core region" (which is also referred to as the "core region") and the "edge region." The "core region" is comprised of the amino acid residues located at positions **a** and **d** in the repeating heptad sequence (the "core residues"), while the "edge region" which is immediately adjacent to the core region, is comprised of the
20 amino acid residues **e** and **g** of the heptad (the "edge residues"). The "exposed region," which faces away from a neighboring α -helix in the supercoil structure as illustrated in Figure 1A, contains the amino acid residues located at positions **b**, **c** and **f** in the heptad (the "exposed residues").

The terms "oligomerization" or "oligomerize" as used herein in reference to
25 polypeptide sequences refer to the non-covalent interaction between polypeptides.

The term "specific oligomerization," "specific binding," "specific pairing," "binding specificity," and "pairing specificity," when made in reference to two protein sequences is herein used to refer to the preferential oligomerization between two
30 protein sequences as compared to the oligomerization between either of these two protein sequences to a third protein sequence. Specific oligomerization may be heterospecific or homospecific. The terms "homospecific oligomerization" and

"homospecificity" as used herein refer to the specific oligomerization between two or more polypeptides having the same amino acid sequence. On the other hand, the terms "heterospecific oligomerization" and "heterospecificity" refer to the specific oligomerization between two or more polypeptides having different amino acid sequences. An amino acid sequence is different from another amino acid sequence if it contains one or more amino acids that are not the same as the amino acids in the other amino acid sequence.

The term "homospecific polypeptide" refers to a polypeptide sequence which preferentially oligomerizes with one or more other polypeptides having the same amino acid sequence as compared to oligomerization with one or more other polypeptides having a different amino acid sequence. In contrast, the term "heterospecific polypeptide" refers to a polypeptide which preferentially binds to other polypeptides of a different amino acid sequence when compared to oligomerization with other polypeptides that have the same amino acid sequence.

The terms "stable," "stable combination" and "stable association" when made in reference to two or more polypeptide sequences refer to the strength of the interaction between the polypeptides. The strength of interaction between polypeptides may be measured, for example, by the "dissociation constant" (K_d). The dissociation constant may be determined using methods known to one of ordinary skill in the art including, but not limited to, melting temperature (T_m) as measured, for example, by urea denaturation, competitive ELISA binding, *etc.*

The term "amino acid" as used herein refers to a hydrophobic, hydrophilic, charged, and/or uncharged amino acids as well as derivatives thereof. A "derivative" of an amino acid is a chemically modified amino acid. Illustrative of such modifications would be replacement of hydrogen by an alkyl, acyl, or amino group, or formation of covalent adducts with biotin or fluorescent groups. Such modifications may be desirable, for example, to bind or detect optically the polypeptide containing the derivative amino acid. Amino acids include biological amino acids as well as non-biological amino acids. The term "biological amino acid" refers to any one of the known 20 amino acids which a cell is capable of introducing into a polypeptide translated from an mRNA. The term "non-biological amino acid" refers to an amino

acid which is not a biological amino acid. Non-biological amino acids are useful, for example, because of their stereochemistry or their chemical properties. The non-biological amino acid norleucine, for example, has a side chain similar in shape t that of methionine. However, because it lacks a side chain sulfur atom, norleucine is less susceptible to oxidation than methionine. Other examples of non-biological amino acids include aminobutyric acids, norvaline and allo-isoleucine, which contain hydrophobic side chains with different steric properties as compared to biological amino acids.

Hydrophobic amino acids are less soluble in water than hydrophilic amino acids. "Hydrophobic amino acids" include alanine, valine, leucine, isoleucine, proline, phenylalanine, tyrosine, tryptophan, methionine, aminobutyric acid, norvaline and allo-isoleucine. "Hydrophilic amino acids" include glycine, serine, threonine, tyrosine, asparagine, and glutamine. A "charged amino acid" is an amino acid which contains a net positive charge or a net negative charge. "Positively charged amino acids", which are also referred to as "basic amino acids," include lysine, arginine, and histidine. "Negatively charged amino acids," which are also referred to as "acidic amino acids," include aspartic acid and glutamic acid.

The term "derivative" when used in reference to a nucleic acid refers to the chemical modification of the nucleic acid. Illustrative of such modifications would be replacement of hydrogen by an alkyl, acyl, or amino group. A "derivative" when made in reference to a nucleic acid sequence refers to a nucleic acid sequence which contains one or more derivative nucleic acids. The term "derivative" as used herein in reference to a polypeptide sequence refers to a polypeptide sequence which contains one or more derivative amino acids.

The term "hetero-oligomer" when used in reference to polypeptides refers to a composition which contains two or more polypeptides, where the amino acid sequence of at least one polypeptide is different from the amino acid sequence of another polypeptide, and where the polypeptides interact via non-covalent bonds. A hetero-oligomer may be a heterodimer, heterotrimer, heterotetramer, *etc.* Heterodimers contain two polypeptides, heterotrimers contain three polypeptides, whereas heterotetramers contain four polypeptides.

The term "homo-oligomer" as used herein in reference to polypeptides refers to a composition which contains two or more polypeptides, where the amino acid sequence of each and every polypeptide is the same, and where the polypeptides interact non-covalently. A homo-oligomer may be a dimer, trimer, tetramer, *etc.*

5 The term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed using a recombinant DNA molecule.

10 As used herein, the terms "vector" and "vehicle" are used interchangeably in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another.

The term "expression vector" or "expression cassette" as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism. Nucleic acid sequences necessary for expression in prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals.

15 The terms "in operable combination", "in operable order" and "operably linked" as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

20 The term "transfection" as used herein refers to the introduction of foreign DNA into cells. Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, biolistics (*i.e.*, particle bombardment) and the like.

As used herein, the term "probe" when made in reference to an oligonucleotide (*i.e.*, a sequence of nucleotides) refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, recombinantly or by PCR amplification, which is capable of hybridizing to another oligonucleotide of interest. A probe may be single-stranded or double-stranded. Probes are useful in the detection, identification and isolation of particular gene sequences. Oligonucleotide probes may be labelled with a "reporter molecule," so that the probe is detectable using a detection system. Detection systems include, but are not limited to, enzyme, fluorescent, radioactive, and luminescent systems.

The term "probe" when used in reference to a polypeptide refers to a polypeptide which is capable of interacting with another polypeptide of interest. Polypeptide probes may be produced synthetically or by recombinant means. Chemicals methods for polypeptide synthesis are known in the art, such as those described by Cartuthers *et al.*, (1980) Nuc. Acids Res. Symp. Ser 215-23, and by Horn *et al.* (1980) Nuc. Acids Res. Symp. Ser 225-32. Alternatively, a polypeptide probe may be produced by expression in an expression vector of polynucleotides (*i.e.*, a sequence of nucleotides) encoding the probe. Probes are useful in the detection, identification and isolation of particular polypeptide sequences. It is contemplated that the polypeptide probes of the invention may be, but are not necessarily, used in combination with a label. The polypeptide probes of the present invention may be labelled with any "reporter molecule," so that the polypeptide reporter is detectable in any detection system, including, but not limited to enzyme (*e.g.*, ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. It is not intended that the present invention be limited to any particular detection system or label.

The polypeptides of the present invention may also be used for the isolation of a polypeptide of interest. For example, the polypeptide probe may be fused to another molecule capable of binding to a ligand, and the polypeptide isolated by binding the fused composition to the ligand. The ligand may be immobilized to a solid support to facilitate isolation of the fused polypeptide. Ligand-binding systems useful for the isolation of polypeptides are commercially available and include, for example, the

staphylococcal protein A and its derivative ZZ (which binds to human polyclonal IgG), histidine tails (which bind to Ni^{2+}), biotin (which binds to streptavidin), maltose-binding protein (MBP) (which binds to amylose), glutathione S-transferase (which binds to glutathione), *etc.* It is not intended that the polypeptide probes of the present invention be limited to any particular isolation system.

The term "sample" as used herein is used in its broadest sense and includes environmental and biological samples. Environmental samples include material from the environment such as soil and water. Biological samples may be animal or plant, including, human, fluid (*e.g.*, blood, plasma and serum), solid (*e.g.*, stool), tissue, liquid foods (*e.g.*, milk), and solid foods (*e.g.*, vegetables). A biological sample suspected of containing APC polypeptide may comprise a cell, tissue extract, body fluid, protein (in solution or bound to a solid support such as for Western blot analysis), and the like.

The terms "polypeptide of interest" refer to any polypeptide, the manipulation of which may be deemed desirable for any reason, by one of ordinary skill in the art.

The term "portion" when used in reference to a protein (as in a "portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid residue.

As used herein, "APC coiled coil probe" or "APC coiled coil polypeptide probe" refers to the amino acid sequence of a substantially purified polypeptide which comprises a coiled coil region, wherein the coiled coil region is capable of specific oligomerization with an adenomatous polyposis coli (APC) protein obtained from any species (particularly mammalian, and preferably human) from any source whether natural, synthetic, semi-synthetic or recombinant.

A "variant" when made in reference to a polypeptide sequence is defined as an amino acid sequence which differs by one or more amino acids from the polypeptide sequence.

The term "biologically active" when made in reference to a coiled coil polypeptide refers to a coiled coil polypeptide molecule which is capable of specific oligomerization with another coiled coil polypeptide molecule.

As used herein, the term "substantially purified" refers to molecules, either nucleic or amino acid sequences, that are removed from their natural environment, isolated or separated, and are at least 60% free, preferably 75% free, and most preferably 90% free from other components with which they are naturally associated.

5 An "isolated polynucleotide" is therefore a substantially purified polynucleotide.

"Amplification" is defined as the production of additional copies of a nucleic acid sequence and is generally carried out using polymerase chain reaction technologies well known in the art [Dieffenbach CW and GS Dveksler (1995) *PCR Primer, a Laboratory Manual*, Cold Spring Harbor Press, Plainview NY]. The term
10 "polymerase chain reaction" ("PCR") refers to the method of K.B. Mullis, U.S. Patent Nos. 4,683,195 and 4,683,202, hereby incorporated by reference.

The term "hybridization" as used herein refers to any process by which a strand of nucleic acid joins with a complementary strand through base pairing.

As used herein the term "hybridization complex" refers to a complex formed
15 between two nucleic acid sequences by virtue of the formation of hydrogen bonds between complementary G and C bases and between complementary A and T bases;
these hydrogen bonds may be further stabilized by base stacking interactions. The two complementary nucleic acid sequences hydrogen bond in an antiparallel configuration. A hybridization complex may be formed in solution (*e.g.*, C_0t or R_0t
20 analysis) or between one nucleic acid sequence present in solution and another nucleic acid sequence immobilized to a solid support [*e.g.*, a nylon membrane or a nitrocellulose filter as employed in Southern and Northern blotting, dot blotting or a glass slide as employed in *in situ* hybridization, including FISH (fluorescent *in situ* hybridization)].

25 As used herein, the terms "complementary" or "complementarity" when used in reference to polynucleotides refer to polynucleotides which are related by the base-pairing rules. For example, for the sequence 5'-AGT-3' is complementary to the sequence 5'-ACT-3'. Complementarity may be "partial," in which only some of the nucleic acids' bases are matched according to the base pairing rules. Or, there may be
30 "complete" or "total" complementarity between the nucleic acids. The degree of complementarity between nucleic acid strands has significant effects on the efficiency

and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, as well as detection methods which depend upon binding between nucleic acids.

The term "homology" when used in relation to nucleic acids refers to a degree of complementarity. There may be partial homology or complete homology (*i.e.*, identity). A partially complementary sequence is one that at least partially inhibits a completely complementary sequence from hybridizing to a target nucleic acid is referred to using the functional term "substantially homologous." The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or Northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (*i.e.*, the hybridization) of a completely homologous to a target under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (*i.e.*, selective) interaction. The absence of non-specific binding may be tested by the use of a second target which lacks even a partial degree of complementarity (*e.g.*, less than about 30% identity); in the absence of non-specific binding the probe will not hybridize to the second non-complementary target.

Low stringency conditions when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42°C in a solution consisting of 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH₂PO₄•H₂O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.1% SDS, 5X Denhardt's reagent [50X Denhardt's contains per 500 ml: 5 g Ficoll (Type 400, Pharmacia), 5 g BSA (Fraction V: Sigma)] and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 5X SSPE, 0.1% SDS at 42°C when a probe of about 500 nucleotides in length is employed.

High stringency conditions when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42°C in a solution consisting of 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH₂PO₄ H₂O and 1.85 g/l

EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5X Denhardt's reagent and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 0.1X SSPE, 1.0% SDS at 42°C when a probe of about 500 nucleotides in length is employed.

5 When used in reference to nucleic acid hybridization the art knows well that numerous equivalent conditions may be employed to comprise either low or high stringency conditions; factors such as the length and nature (DNA, RNA, base composition) of the probe and nature of the target (DNA, RNA, base composition, present in solution or immobilized, etc.) and the concentration of the salts and other
10 components (e.g., the presence or absence of formamide, dextran sulfate, polyethylene glycol) are considered and the hybridization solution may be varied to generate conditions of either low or high stringency hybridization different from, but equivalent to, the above listed conditions. The term "hybridization" as used herein includes "any process by which a strand of nucleic acid joins with a complementary
15 strand through base pairing." [Coombs J (1994) *Dictionary of Biotechnology*, Stockton Press, New York NY].

 "Stringency" when used in reference to nucleic acid hybridization typically occurs in a range from about $T_m - 5^\circ\text{C}$ (5°C below the T_m of the probe) to about 20°C to 25°C below T_m . As will be understood by those of skill in the art, a stringent
20 hybridization can be used to identify or detect identical polynucleotide sequences or to identify or detect similar or related polynucleotide sequences. Under "stringent conditions" SEQ ID NO: or fragments thereof will hybridize to its exact complement and closely related sequences. The stringent conditions are chosen such that SEQ ID NO: or fragments thereof will hybridize to sequences encoding the APC polypeptide
25 of SEQ ID NO:

GENERAL DESCRIPTION OF THE INVENTION

 The present invention solves the problems in the prior art through the provision of a polypeptide probe system directed specifically to the coiled coil region of a target protein. The coiled coil region is a widely-distributed protein motif
30 containing a seven-residue repeat labelled (a b c d e f g)_n, with hydrophobic residues

generally occurring at the **a** and **d** positions and largely polar residues elsewhere. This repeating heptad forms a right-handed α -helix, which is capable of wrapping around an opposing coiled coil region in a left-handed supercoil, provoking the name "coiled coil." As illustrated in Figure 1, the **a** and **d** positions typically comprise the hydrophobic core of the interhelical interface, with the charged **e** and **g** residues on the edge of the interhelical interface adjacent to the core.

In order to provide an effective biological probe, the hetero-oligomer formed by the target and probe polypeptides should be more stable than either homo-oligomer. The methods described herein provide a polypeptide probe sequence with high affinity for any desired coiled coil region, effectively competing with the natural pairing. The invention contemplates selective amino acid pairing and packing in the hydrophobic core of the interhelical interface, as well as optimization of the electrostatic interactions in the amino acid residues surrounding the hydrophobic core.

Thus, the methods of the present invention can be used to provide heterospecific polypeptide probes based solely on the amino acid sequence of the target protein. Since coiled coil regions can be found in a multitude of important proteins, including, but not limited to, cytoskeletal components, transcriptional activators, tumor suppressors, components of the mitotic apparatus, nuclear envelope proteins, extracellular matrix components, oncogene products, muscle proteins, cell and viral surface proteins and molecular motors, it is contemplated that the polypeptide probes of the present invention will have broad diagnostic and therapeutic application.

I. Coiled Coil Proteins

The coiled coil has a distinguished history. In 1952, Crick and Pauling raced to propose the coiled-coil structure to reconcile Pauling's model of the α -helix with the diffraction patterns of a variety of fibrous proteins, including keratin. Crick, *Nature* 170:882-83 (1952); Pauling and Corey, *Nature* 171:59-61 (1953). Importantly, in 1953 Crick proposed a detailed mathematical model of the coiled coil family in which the characteristics of the supercoil (radius, pitch, helix crossing angle, etc.) were shown to be dependent on each other. Crick, *Acta Cryst.* 6:685-89 (1953).

Crick also described a physical model (based on the packing of nails hammered into a broom handle) for the packing of hydrophobic side chains in the interface between the helices. Crick, *Acta Cryst.* 6:689-97 (1953). The essence of this model, which Crick termed "knobs-into-holes" packing, was that each core side chain (the knob) would fit in the space (the hole) between four side chains in the neighboring helix. This packing model is considered to be the hallmark of a true coiled coil structure. See, e.g., Lupas, *TIBS* 21:375-82 (1996).

As noted above, the sequences of coiled coil proteins revealed a common seven-residue repeat, (a b c d e f g)_n, with the first (i.e., a) and fourth (i.e., d) positions generally occupied by hydrophobic residues. The occurrence of hydrophobic residues spaced every three and then four residues apart is a characteristic of all coiled coil sequences, and the sequence is strongly maintained for structural reasons. The seven helical residues sweep out 700°, 20° short of two full turns. As a result, succeeding hydrophobic residues wind gently around the helix, and the coiled coil forms when the hydrophobic stripes associate. The coiled coil family now includes seven structural classes--dimers, trimers, tetramers with parallel or antiparallel helices, as well as a pentamer with parallel helices. See Wagner and Green, *Science* 262:395-99 (1993); (Table 1).

The number of proteins found to contain coiled-coil motifs has grown in equal measure to the sequence data base. Cohen and Parry, *Proteins* 7:1-15 (1990). Coiled-coil proteins include the transcription factors and oncogene products in the bZIP and bHLH-ZIP families, as well as tumor suppressors, such as APC. Longer coiled coils occur in structural proteins of the cytoskeleton, nuclear matrix, mitotic spindle, molecular motors, muscle, extracellular matrix and surface proteins of cells and viruses, and the methods and probes of the present invention can be used to detect these target coiled coil proteins either *in vitro* or *in vivo*, or alternatively to differentiate the tissue and/or the cells expressing the target protein when it is expressed exclusively by that tissue and/or cell type. In addition to providing oligomerization domains and spacers, coiled coils are bound by other proteins (Wagner (1993). The following Table provides an illustrative example of the rich

variety of coiled coil proteins which can be analyzed and manipulated using the methods and probes provided by the present invention.

TABLE 1

COILED COIL PROTEINS				
Orientation	Number of α -helices			
	TWO	THREE	FOUR	FIVE
PARALLEL	Leucine zipper APC myosins tropomyosins Intermediate Filament proteins	Hemagglutinin Heat Shock Factor Laminin Fibrinogen Phage T7 leg Tenascin	GCN4-pLI	Cartilage Oligomer Matrix Protein
ANTI-PARALLEL	Seryl tRNA synthetase Serum Response Factor	Spectrin Coil-Ser	Repressor of Primer LacI	

Published reports in the literature describing specific examples of coiled coil proteins include Wilson *et al.*, *Nature* 289:366 (1981) (hemagglutinin); Banner *et al.*, *J. Mol. Biol.* 196:657-75 (1987); O'Shea *et al.*, *Science* 254:539-44 (1991); Cohen & Parry, *supra*; Cusack *et al.*, *Nature* 347:249-55 (1990) (seryl-tRNA synthetase); Beck *et al.*, *J. Mol. Biol.* 231:311-23 (1993) (laminin); Lovejoy *et al.*, *Science* 259:1288-93 (1993); Yan *et al.*, *Science* 262:2027-30 (1993) (spectrins); Holberton *et al.*, *J. Mol. Biol.*, 204:789-95 (1988) (giardin); Koski *et al.*, *J. Biol. Chem.*, 267:12258-65 (1992) (the protein TlpA from *Salmonella typhimurium*); Ayer *et al.*, *Cell* 72:211-22 (1993) (Myc oncoprotein Max); Gauger & Goldstein, *J. Biol. Chem* 268:13657-66 (1993) (*Drosophila* kinesin light chain); Marshal & Holberton, *J. Mol. Biol.* 231:521-530 (1993); and Zervos *et al.*, *Cell* 72:223-32 (1993) (Mxi1). In addition, coiled coil databases have been constructed and analyzed to verify the existence of coiled coil

regions in a multitude of proteins. See, e.g., Woolfson and Alber, *Protein Science* 4:1596-1607 (1995); Berger *et al.*, *Proc. Natl. Acad. Sci. USA* 92:8259-63 (1995).

The methods of this invention contemplate the use of coiled coil polypeptides which are capable of hetero-oligomerization with any desirable target polypeptide which contains a heptad repeat. The methods of this invention are not limited to the number of polypeptides in the target coiled coil polypeptide. For example, the target coiled coil polypeptides may comprise two, three, four, five, or more polypeptides (see, e.g., Table 1). Examples of coiled coil proteins which contain two polypeptides include the β -isoform of human cardiac, heavy-chain myosin; human tropomyosin, α and γ chains; paramyosin; human desmoplakins I and II; the transcriptional activator, GCN4; human cytokeratins 1 and 10; and the N-terminal 80 amino acids of the human adenomatous polyposis coli (APC) protein, etc. Examples of coiled coil polypeptides which comprise three polypeptides include human laminins A, B1 and B2; the B chain of influenza hemagglutinin; the trimerization region of the human heat shock transcription factor, hsf2; human fibrinogen, β and γ chains; the human macrophage scavenger receptor; the DNA packaging protein, GP17; human tenascin; the whisker antigen control protein, fibrin, etc.

The invention further contemplates coiled coil proteins comprising the same or different amino acid sequences. Also expressly included within the invention's scope are coiled coil polypeptides in which the α -helices run in the same (parallel) or in opposite (antiparallel) directions.

Also contemplated within the scope of this invention are coiled coiled regions which contain discontinuities in the heptad repeat, including, but not limited to, non-helical regions, skip residues, and omission of either three or four residues from the heptad repeat. "Non-helical regions" are amino acid sequences which do not contain a heptad repeat, and they generally give the coiled coil region in which they are located the appearance of a segmented rope. A "skip residue" refers to a coiled coil region in which one or more heptad repeats contains one or more extra amino acid residues (e.g. *abcdeffg*). The skip residue structures are observed, for example, in the 'thumb' of DNA polymerase 1 (Poll). "Omissions of three residues" and "stutters" refer to the absence of three amino acid residues from the heptad repeat. These cause a local

decrease in the extent of supercoiling as they correspond to slightly less than a full turn of the helix. Stutters shift residues in position *a* towards the center of the core region, resulting in a geometry called an *x* layer (*i.e.*, *abcdefgxelg*). "Omissions of four residues" refers to the absence of four amino acid residues from the heptad repeat. These cause an increase in supercoiling as they correspond to slightly more than a helix turn. Their effect is to shift residues in *a* into a *da* layer (*abcedfngelg*) and residues in *d* into an *x* layer (*i.e.*, *abcxbcdelg*).

II. Specificity of Coiled Coil Binding

Despite having a common structure, the hundreds of coiled coil motifs within a cell generally exhibit specific binding preferences. Thus, while sharing a common structure, dimeric coiled coils pair specifically. O'Shea, *Cell* 68, 699-708 (1992). This specificity has been demonstrated in wild-type protein sequences (Lavigne, *et al.*, *J. Mol. Biol.* 254, 505-20 (1995); Kammerer *et al.*, *J. Engel, J. Mol. Biol.* 250, 64-73 (1995); O'Shea, *Science* 245, 646-8 (1989)) and synthetic model systems (Graddis *et al.*, *Biochemistry* 32, 12664-71 (1993); Hodges *et al.*, *J. Biol. Chem.* 256, 1214-1224 (1981); Nautiyal *et al.*, *Biochemistry* 34, 11646-11651 (1995).

A clear example of this specificity is afforded by the oncogene products Fos and Jun, whose leucine zippers (a classic coiled coil sequence having a leucine every seven residues) have been found to mediate preferential pairing with each other to form a heterodimer. See, *e.g.* O'Shea *et al.* (1989); O'Shea *et al.* (1991); Alber, *Curr. Opin. Genet. Dev.* 2:205-10 (1992). Subsequent experiments with the Fos-Jun model demonstrated that the *e* and *g* positions of the coiled coil heptad were determinative of this specificity, since repulsive ionic interactions between the *e* and *g* residues of Fos were relieved by complementary charges in the heterodimer. O'Shea *et al.* (1992). Importantly, these investigators also found that substitution of the *a* and *d* residues in the hydrophobic core did little to alter the preference for heterodimer formation. See, *e.g.*, Figure 5 of O'Shea *et al.* (1992) *supra*. Based on these studies, the investigators concluded that the major thermodynamic driving force for preferential heterodimer formation was the destabilizing electrostatic interactions at the *e* and *g* positions in the edge region adjacent to the hydrophobic core.

This analogous charge complementation at the e and g positions has formed the basis for the design of synthetic heterodimers, and for peptides that bind to specific leucine zipper targets. See, e.g., O'Shea *et al.*, *Current Biol.* 3(10):658-67 (1993); Zhou *et al.*, *J. Mol. Biol.* 237:500-12 (1994); Krylov *et al.*, *EMBO J.* 13:2849-61 (1994). In contrast to modifications of the e and g residues, prior art attempts to design heterodimeric coiled coils with alternative core residue packing in the interhelical interface did not result in heterodimers that were more stable than the corresponding homodimers. See Zhu *et al.*, *Int. J. Pept Protein Res.* 40:171-79 (1992). Thus, the prior art has focused almost exclusively on optimizing the electrostatic interactions at the e and g positions of the coiled coil heptad in order to drive oligomer heterospecificity.

In contrast, the methods of the present invention focus in significant part on optimal amino acid residue pairing in the core region of the interhelical interface. To date, these interactions have been far less well understood. See Lupas, *TIBS* 21, 377 (1996). However, the preferred embodiments of the methods of the present invention provide a solution to this persistent problem in the art, through the provision of core residue pairing rules which optimize the packing and polarity of the core amino acids located at the a and d positions in the heptad. In addition, a further embodiment of the present invention also incorporates the optimization of the electrostatic interactions in the edge regions of opposing helices, to further enhance the heterospecificity of the polypeptide.

To develop pairing rules that could guide the design of antisense coiled-coil peptides, we relied on thermodynamic measurements of specificity (O'Shea (1992); Vinson *et al.*, *Genes Dev.* 7, 1047-58 (1993).; Krylov *et al.*, *EMBO J.* 13, 2849-2861 (1994)) and covariation frequencies in coiled-coil sequences (Fig. 1). In both natural and model coiled coils, specific associations have been attributed to destabilizing ionic interactions in the homodimers that are replaced with more favorable contacts in the heterodimer (O'Shea (1992); Vinson *et al.* (1993).; Krylov *et al.* (1994); Zhou *et al.*, *Prot. Eng.* 7, 1365-1372 (1994)). Consequently, our anti-APC design included charged amino acids at g and succeeding e' positions that could form ion pairs in the heterodimer and repulsive ionic interactions in the antisense peptide (Vinson (1993)).

In contrast to the g and e positions, little specificity has been ascribed to core residue contacts (O'Shea (1992); Lavigne (1995), Lumb *et al.*, *Biochemistry* 34, 8642-8 (1995); Zeng *et al.*, *Proc. Natl. Acad. Sci. USA* 94, 3673-3678 (1997)). In the heterodimer formed by the Fos and Jun leucine zippers, for example, three heterotypic
5 pairs of core residues appear to impart none of the observed preference of the chains for each other (O'Shea (1992)). This is surprising, considering that the core residues form the majority of interhelical contacts (O'Shea (1991), and mutations of core residues have dramatic effects on stability and oligomerization (Monera *et al.*, *Prot. Eng.* 9, 353-63 (1996); Harbury *et al.*, *Science* 262, 1401-7 (1993); Harbury *et al.*,
10 *Nature* 371, 80-83 (1994); Lovejoy *et al.*, *Science* 259, 1288-93 (1993); Vieth *et al.*, *J. Mol. Biol.* 251, 448-67 (1995); Zhu *et al.*, *Prot. Sci.* 2, 383-94 (1993)).

To obtain rules for core complementation, we analyzed the sequences of the coiled-coil domains in cytokeratins that form obligate heterodimers (Corden *et al.*,
Exp. Dermatol. 5, 297-301 (1996); Steinert *et al. Ann Rev. Cell Biol.* 1, 41-65 (1985)).

15 Four pairs (comprising four known keratin sequences) were used in the analysis. Sequences are available in the Swissprot database with the following accession numbers: K8 (P05787), K18 (P05783), K4 (P19013), K13 (P13646), K5 (P13647), K14 (P02533), K1 (P04264), and K10 (P13645). Uneven frequencies of heterotypic and homotypic pairing provided evidence for core mediated specificity and revealed
20 the identities of preferred interhelical neighbors.

III. Applications of The Present Invention

Because coiled coils are central to many areas of biology, the new technology provided by the present invention has a variety of advantageous uses. These include, for example, staining target proteins on gels and in cells, inactivating coiled coils *in vivo*, affinity purification of a target protein as well as coprecipitation and
25 identification of any associated proteins, tagging cells for sorting, disassembling viruses, screening tissue for mutant forms of a protein, developing drugs that block oligomerization, and diagnosing human diseases such as colon cancer. Fusion to sequences that target proteins for degradation also ultimately will allow peptide
30 probes to reduce the level of specific target proteins *in vivo*.

Additional applications for peptide probe technology are also contemplated. Probes against any coiled coil proteins may be designed using this approach, and used to detect target coiled coil proteins or alternatively to differentiate tissue and/or cells expressing a tissue- or cell-specific target protein from surrounding tissue, such as for example in histological staining. Moreover, the probes may prove suitable for expression alone or as fusion proteins within cells, because unlike antibodies, the peptide probes do not require disulfide bonds for structural stability. Also unlike antibodies, which can recognize the native structure of their target, peptide probes for coiled coils necessarily disrupt the structure of the target coiled coil. As a result, the peptide probe can unfold and inactivate the target. This property is useful in applications in research and medicine.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

As noted above, the methods of the present invention contemplate the creation of a heterospecific polypeptide probe, by focusing on both packing and preferential pairing in the hydrophobic core between the opposing helices, and on optimizing electrostatic interactions in the edge region adjacent to the core.

I. Sequence Alterations Contributing to Heterospecificity

A. The Core Residue Packing Rules

The methods and probes of the present invention contemplate the incorporation of core residues into the probe polypeptide to conserve packing volume and, in a preferred embodiment, to duplicate a-a' and d-d' pairs that occur with enhanced frequency in the sequences of obligate heterodimeric coiled coils. In the preferred embodiment, the sequences of suitable obligate heterodimers are analyzed in accordance with the disclosed method to identify preferred heteromeric pairs of amino acids. Once the sequence of the target coiled coil region has been determined, these pairing preferences are used to identify and incorporate the optimal amino acid into the opposing position in the core region of the probe polypeptide. Thus, the pairing preferences provided by the present invention provide an initial set of empirical rules

for complementation in the core of the interhelical interface between opposing coiled coil regions.

In a particularly preferred embodiment, the polypeptide probes of the present invention are constructed in accordance with the pairing frequencies found in keratin heterodimers. In keratins, small residues in the core region of one helix are generally paired with larger side chains in the core of the opposing helix. In addition, polar residues in the interhelical interface are generally paired with polar residues in the opposing interface. These general principles, along with the preferred heteromeric pairs derived from analysis of the keratin sequences, are employed in the preferred embodiments of the methods of the present invention to create a heterospecific polypeptide probe directed to any target coiled coil region.

B. Optimizing Electrostatic Interactions In The Edge Region

The methods and probes of the present invention also contemplate the optimization of electrostatic interactions in the edge region of the coiled coil, as disclosed to some extent in the prior art. Thus, the polypeptide probes of the present invention preferably also incorporate repulsive charge / charge interactions at position g and position e of the succeeding heptad to disfavor homo-oligomerization of the polypeptide probe, as well as complementary charges in comparison with the opposing edge region in the target polypeptide in order to aid in favorable interhelical ionic interactions in the hetero-oligomer.

II. Sequence Alterations Not Contributing To Heterospecificity

The present invention further contemplates that additional advantageous sequence alterations can be made in the probe polynucleotide sequence which are not considered to influence heterospecific oligomerization. For example, several alterations can be made at the (f) position in the heptad in order to 1) change the overall charge of the probe in order to facilitate experimental analysis of pairing; 2) preserve the overall stability of the system by incorporating a residue with significant helical propensity, such as Lysine or Alanine; 3) promote the solubility of the probe peptide; and 4) introduce chromogenic groups (*e.g.* Tyrosine) to allow the

probe to be detected optically. Other and further advantageous alterations, known to those of skill in the art, can be employed where appropriate to the specific target protein and/or application contemplated for a given probe polypeptide.

III. Generation of A Coiled Coil Polypeptide

5 Coiled coil polypeptides may be made using any of several methods known in the art including synthetic and recombinant methods. Chemicals methods for polypeptide synthesis are known in the art, such as those described by Caruthers *et al.*, (1980) Nuc. Acids Res. Symp. Ser 215-23, and by Horn *et al.*, (1980) Nuc. Acids Res. Symp. Ser 225-32. Automated chemical protein synthesis is routine in the art, for
10 example using "Fast Moc" chemistry on an Applied Biosystems model 431A peptide synthesizer. Briefly, amidated peptide may be prepared using Rink resin (Advanced ChemTech), whereas peptide containing free C termini may be synthesized on Wang (*p*-alkoxybenzyl alcohol) resin (Bachem). First, residues are double coupled to the appropriate resin and subsequent residues are single coupled. Each coupling step is
15 followed by acetic anhydride capping. Peptide are cleaved from the resin by treatment with trifluoroacetic acid (10 ml), water (0.5 ml), thioanisole (0.5 ml), ethanedithiol (0.25 ml) and crystalline phenol (0.75 ml). Purification may be carried out by reverse-phase HPLC. Samples of crude peptide may be chromatographed on a Waters Delta Pak C18 column with a linear gradient as described by Cionciolo, *et al.*
20 (1988) *Immunol. Lett.* 19:7-14. Lyophilized peptide may be stored either desiccated or in an aqueous solution. Electrospray mass spectrometry may be used to confirm the purity and size of the synthesized coiled coil polypeptide.

Alternatively, a coiled coil polypeptide may be produced by expression in an expression vector of polynucleotides (*i.e.*, a sequence of nucleotides) encoding the
25 coiled coil polypeptide. For example, the nucleic acid sequence encoding the coiled coil polypeptide may be expressed either alone or as a fusion polypeptide ligated to one or more heterologous sequences as part of a fusion gene. Fusion genes may be desirable, for example, for the detection or the purification of the coiled coil protein. Examples of a heterologous sequence useful for the detection of the expression of the
30 coiled coil polypeptide include the reporter sequence encoding the enzyme

5 β -galactosidase or the enzyme luciferase. Fusion genes desirable to facilitate purification of the expressed coiled coil polypeptide include the protein A which allows purification of the fusion protein on immobilized immunoglobulin. Other affinity traps are well known in the art and can be utilized to advantage in purifying the expressed fusion protein. For example, pGEX vectors (Promega, Madison WI) may be used to express the coiled coil polypeptide as a fusion protein with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Other fusion polypeptides useful in the purification of the coiled coil polypeptide are commercially available, including histidine tails (which bind to Ni^{2+}), biotin (which binds to streptavidin), and maltose-binding protein (MBP) (which binds to amylose). It is not intended that the coiled coil polypeptides of the present invention be limited to any particular isolation system. Coiled coil polypeptides made in such systems are designed to include heparin, thrombin or factor XA protease cleavage sites so that the cloned polypeptide of interest can be released at will from the heterologous polypeptide moiety to which it is fused.

IV. Methods For Verifying Heterospecificity And Stability In The Probe Polypeptide

20 Coiled coil polypeptides are useful in the detection, identification and isolation of particular polypeptide sequences, and in detecting and differentiating the cells and/or tissues which express these proteins. These uses are feasible as a result, in part, of the heterospecificity of the coiled coil polypeptides for other polypeptides containing coiled coil regions, and the stability of the formed hetero-oligomers. The ability of the methods of the present invention to create a stable, heterospecific polypeptide probe can be easily demonstrated using standard biochemical techniques known to those of skill in the art. As an initial approach, heterospecificity of a coiled coil polypeptide is determined prior to the quantitation of heterospecificity. Heterospecificity is determined using a number of methods known in the art, including, but not limited to, circular dichroism, thermal denaturation, equilibrium

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centrifugation, direct measurement of dissociation constants, disulfide exchange measurements, ion exchange chromatography, western blot analysis, precipitation of target proteins, biological screening methods, and native gel electrophoresis.

Circular dichroism investigations are well known in the art. For example, such an investigation may be performed using a 1 cm or 1 mm cuvette (Helma or Uvonic) on an Aviv CD spectrophotometer (model 60DS or model 62DS) equipped with a thermoelectric controller. The buffer used for all CD experiments except pH titrations is 100 mM KF, 10 mM potassium phosphate (pH 7.0). All peptide concentrations may be determined by tyrosine absorbance at 275.5 nm in 6.0 M GuHCl PH 6.5 (Schwarz/Mann Biotech Ultra-Pure grade) using an Aviv UV/VIS spectrophotometer (model 1 BDS or 14 DS). The molar ellipticity at 222 nm, 5°C, is measured for the homo- and heterodimers. The CD values (*i.e.*, deg cm² and dmol⁻¹) indicate % helicity of the peptides. Thermal melting curves are determined by monitoring the CD signal at 222 nm as a function of temperature. The pH dependence of stability may be measured in 100 mM KF, 10 mM potassium phosphate at various pH values. GuHCl (Schwarz/Mann-Biotech-Ultra-Pure grade) (e.g., 2M) may be included in the buffer used for monitoring the pH dependence of stability. pH stability is indicated if the same overall shape of the pH dependence curve is obtained in the absence as well as the presence of GuHCl. It is also desirable to determine the reversibility for all thermal melts. The absence of reversibility of a melting curve above a certain pH value indicates chemical modification and/or degradation of the polypeptide. Such modification and/or degradation may be confirmed using HPLC.

Thermal denaturation is used to determine T_m . The term " T_m " refers to the temperature at which the fraction of a polypeptide which is unfolded is equal to the fraction folded ($\Delta G = 0$). The T_m is determined by curve fitting a thermal denaturation curve (described *supra*) to an equation known in the art (*see*, O'Shea *et al* (1992) Cell 68:699-708) using a nonlinear least squares-fitting program (e.g., Kaleidagraph, Synergy Software). Alternatively, the T_m for each polypeptide may also be determined by taking the first derivative of the CD signal (β) with respect to temperature⁻¹ (temperature in K) and finding the minimum of this function (Cantor and Schimmel, 1980). Similarly, denaturation induced by additives such as urea or

GuHCl can be followed by circular dichroism. These measurements can yield the C_m , i.e., the denaturant concentration where $\Delta G=0$. Linear extrapolation of the transition to zero denaturant can yield the equilibrium constant in aqueous buffer. This can be converted to a free energy of association using the formula $\Delta G = -RT \ln K$ [Cantor and Schimmel (1980) *supra*].

Equilibrium centrifugation is yet another routinely used alternative method for the determination of the specificity of hetero-oligomerization. Briefly, this method involves ultracentrifugation of different concentrations of each peptide alone and in combination, analyzing samples for the presence of protein (e.g., by measuring absorbance at 280 nm), and determination of partial specific volumes based on the amino acid compositions of the peptide and apparent molecular weights using commercially available software.

Direct measurement of dissociation constants (K_d 's) may also be used to determine the specificity of polypeptide hetero-oligomerization and may be accomplished using any number of quantitative binding methods. These methods include, but are not limited to, fluorescence, plasmon resonance, gel shifts, competition ELISA and mass spectrometry.

Disulfide exchange measurements are also routinely used in the art to determine the specificity of polypeptide hetero-oligomerization. Polypeptide variants capable of forming a disulfide bond in the folded structures of all possible peptide combinations are mixed in a redox buffer. The redox buffer, consisting for example of a mixture of reduced and oxidized glutathione, allows assortment of the disulfide bonded species. After equilibrium is reached, the disulfide exchange is quenched at low pH and the ratio of oxidized species is determined. Deviations from the expected 1:2:1 binomial distribution quantitatively indicate specificity if the heterodimer is enriched and anti-specificity if the homodimers are enriched. E.K. O'Shea, R. Rutkowski, W.F. Stafford III and P.S. Kim (1989), *Science*, 245: 646-648.

Yet another method for the determination of hetero-oligomerization specificity is ion exchange chromatography which is capable of resolving polypeptide species with different charges (Nautiyal *et al.* (1995). A further method for determining the specificity of hetero-oligomerization is by using the coiled coil polypeptide probe

whose specificity is sought to be determined to bind to Western blots of cell lysates or other mixtures of proteins. Exclusive binding to a target protein indicates a preference or specificity for the target.

Additionally, precipitation of target proteins may be used to determine the hetero-oligomerization specificity of a coiled coil polypeptide probe to the target proteins. Coupling the probe polypeptide to biotin, GST, His tag or other epitope allows probe-bound complexes to be rapidly removed from cell lysates. Analysis of the targets bound to the probe by SDS gel electrophoresis and Western blotting allows the preference for the target protein to be assessed.

Other biological screening methods, such as phage display of the "two-hybrid" system may also be used to determine the hetero-oligomerization specificity of a coiled coil polypeptide. Expression of the probe sequence (or a portion thereof) on the surface of a filamentous phage might allow specificity to be estimated by panning the phage population using the target polypeptide. Alternatively, specificity might be detected *in vivo* by attaching the coiled coil target polypeptide and the coiled coil probe polypeptide to the GAL4 DNA binding domain and the activation domain in a two hybrid reporter system in yeast. Preferential binding of the probe to the target could lead to increased expression of the reporter gene.

Native gel electrophoresis is the preferred method for the determination of specificity of hetero-oligomerization. In this method, the individual peptides and an equimolar mixture of the peptides are electrophoresed on gels in the absence of denaturing compounds (e.g., urea, SDS, etc.) and the location of the polypeptide bands determined using for example, Coomassie Brilliant Blue. The formation of a band of a different mobility when the two polypeptides are combined and the absence of the bands corresponding to the individual polypeptides indicates heterospecificity.

In addition to the determination of whether or not a coiled coil polypeptide exhibits heterospecificity, the degree of specificity of a coiled coil polypeptide (e.g., a probe) for another coiled coil polypeptide (e.g., a target) can be quantitated using methods known in the art, such as those described by O'Shea *et al.* (1993) Current Biology 3:658-667. Briefly, specificity may be estimated using the equilibrium constant K_{spec} which describes the ratio of heterodimers to homodimers, and which

can be expressed in terms of the dissociation constants for each dimer. Assuming a two-state model for monomer-dimer equilibrium, then

$$K_{\text{spec}} = K_d(\text{AB}) / [K_d(\text{AA})^{1/2} \times K_d(\text{BB})^{1/2}]$$

where $K_d(\text{AB})$, $K_d(\text{AA})$ and $K_d(\text{BB})$ are the dissociation constants of the probe/target heterodimer, the probe homodimer and the target homodimer, respectively.

The dissociation constant for each dimer is determined so that the degree of specificity, ΔG_{spec} , given by $-RT \ln K_{\text{spec}}$, could be estimated. A dissociation constant is determined, for example, by fitting the urea dependent circular dichroism (CD) signal as a function of urea concentration to a monomer-dimer equilibrium. An estimate of the dissociation constant for each homodimer is obtained from measurements of the helical CD signal as a function of peptide concentration.

Alternatively, specificity may also be determined by measuring ΔT_m , i.e., the difference between the T_m of the heterodimer and the average of the T_m values for the homodimers as described by O'Shea *et al.*, (1992) *Cell* 66:699-708. This ΔT_m difference has been measured for disulfide-bonded leucine-zipper peptides and has been shown to be related to ΔG_{spec} by a proportionality constant of 7.4°C/kcal mol⁻¹ (O'Shea *et al.* (1992) *supra*).

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: eq (equivalents); M (Molar); μM (micromolar); N (Normal); mol (moles); mmol (millimoles); μmol (micromoles); nmol (nanomoles); g (grams); mg (milligrams); kg (kilograms); μg (micrograms); L (liters); ml (milliliters); μl (microliters); cm (centimeters); mm (millimeters); μm (micrometers); nm (nanometers); °C (degrees Centigrade); h (hours); min (minutes); sec (seconds); msec (milliseconds); TLC (thin layer chromatography);

EXAMPLE 1

Determination of The Amino Acid Pairing Preferences At Positions **a** And **d** Of The Repeating Heptad In A Coiled Coil Region Using The Coiled Coils In Keratin Proteins

5 In order to determine the pairing preference at positions **a** and **d** in the repeating heptad of a coiled coil region, pairing frequencies at these positions in obligate heterodimers were calculated. This approach was based on the assumption that the four heptad positions that make interhelical contacts in the coiled coil region are most likely to determine pairing specificity. While experiments can be envisioned
10 to measure the free energies of heterotypic contacts, a simpler initial approach of calculating residue pair frequencies at positions **a**, **d**, **e** and **g** in the sequences of heterodimers was first adopted. These pair frequencies were expected to reveal the pairing preferences that form the basis for heterotypic associations.

The number of obligate heterodimer sequences is limited, and those in which
15 the register of the helices is known is even more restricted. The human heterodimers K1-K10, K5-K14, K4-K13, and K6-K16 were selected because they form obligate heterodimers and because the amino acid sequence for each of these proteins is known. In addition, the four distinct coiled-coil domains in the epidermal keratins mediate formation of specific heterodimers, even when multiple keratin sequences are
20 expressed in a particular cell. The sequence homologies between the four coiled-coil segments in each keratin chain allowed the register (*i.e.*, the matrix of pairing frequencies) to be deduced unambiguously.

The amino acid sequences were analyzed by eye and the heptad positions assigned based on the characteristic 3,4 spacing of hydrophobic residues. Three
25 residues separated successive **a** and **d** positions, and four residues separated each **d** position from the next **a** position (the heptad positions in the sequences of type I and type II obligate heterodimers of keratins were subsequently confirmed using the program PAIRCOIL (Berger *et al.* (1995)). To analyze these assignments for preferred heteromeric pairs at the **a**, **d**, **e** and **g** positions, a short awk script was
30 written to calculate the probability that each observed pairing frequency arose by

random association. Segments with different hydrophobic spacings, the so-called stutter motifs, were excluded from the analysis.

By tabulating the frequency of each amino acid contact in the **a**, **d**, **e** and **g** positions, 20 x 20 preference matrices were obtained (Tables 2-5).

TABLE 2
Frequency of a:a' and d:d' Interactions in Human Keratins

	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
Y	4		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
W	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
V	2	0	0		0	0	1	2	0	4	1	0	0	0	0	0	0	1	0	0
T	0	0	1	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S	1	0	0	2	0		0	0	0	26	5	1	0	0	2	2	0	5	3	1
R	0	0	0	0	0	0		9	1	1	1	1	0	0	2	0	0	0	0	0
Q	0	0	3	0	0	0	0		5	14	4	0	0	0	0	2	1	5	0	0
P	1	0	0	0	0	0	0	0		0	2	0	0	0	1	0	0	0	0	0
N	0	0	0	0	0	0	0	0	0	0		4	0	0	3	1	0	0	0	3
M	0	0	3	0	0	0	1	0	0	0	3	51	0	0	0	0	0	0	0	0
L	0	4	6	0	0	1	3	1	1	0	0	0	0	0	0	0	0	1	0	0
K	0	0	0	0	0	0	0	0	0	0	0	0			3	0	0	0	0	0
I	0	0	0	0	1	0	1	0	0	3	3	0	0	0	0	0	0	1	0	1
H	0	0	0	0	0	0	0	0	4	0	0	0	0	0		0	0	0	0	0
G	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	2
F	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3		7	0	0
E	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0	1	9	0	0	0
D	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	10
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A	0	0	3	0	0	0	0	0	3	3	7	1	7	0	0	0	0	0	0	0
Y		W	V	T	S	R	Q	P	N	M	L	K	I	H	G	F	E	D	C	A

Table 2 shows a reference matrix for the amino acids at positions **a** and **d**. The letters A, C, D *etc.* represent the single letter abbreviation of each of the 20 amino acids which were found to be located at the **a**, **a'**, **d**, and **d'** positions. The arabic numerals inside Table 2 represent the frequency at which a combination of two amino acids were found to be located at the **a:a'** positions (right hand triangle of Table 2) and at the **d:d'** positions (left hand triangle of Table 2). Frequencies on the diagonals denote homotypic amino acid pairs in the heterodimer sequences. Amino acids with a high relative frequency of heterotypic pairing and a low relative frequency of homotypic pairing -- such as ala, ile, met and ser at the **a** position and ala, phe, his, ile, met, asn and val at the **d** position -- provide evidence that core residues can confer specificity to coiled-coil dimerization.

Thus, Table 2 shows that certain amino acid residues never pair with like residues in the keratin core sites (e.g. alanine and histidine at the **d** position; alanine and serine at the **a** position). This observation suggested that **a** and **d** residues can influence heterospecificity. The preponderance of leucine:isoleucine and methionine:isoleucine pairs at the **a** positions also suggested that these combinations favored heterodimerization.

The **g:e'** interactions in type I keratin homodimers (Table 3), in type II keratin homodimers (Table 4), and in keratin heterodimers composed of one type 1 and one type 2 chains (Table 5) were compared as to the frequency of repulsive, attractive and hydrogen bonds.

TABLE 3

Frequency of g:e' Interactions in Type I Keratin Homodimers

eg-pair:		A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
5	A	0	0	0	3	0	0	0	5	0	0	0	0	0	5	0	0	1	0	0	0
	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	D	0	0	0	2	0	0	0	0	4	5	0	1	0	4	2	0	0	0	0	5
	E	0	0	1	0	0	0	0	0	24	3	0	8	0	7	2	2	2	0	0	0
	F	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0
	G	0	0	0	0	0	0	0	0	1	5	2	0	0	2	0	1	3	0	0	0
	H	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	I	0	0	0	0	0	0	0	0	12	1	0	1	0	1	2	0	0	0	0	0
	K	0	0	0	0	0	0	0	0	0	4	2	2	0	5	1	2	1	0	0	0
	L	0	0	0	0	0	0	0	0	2	0	0	0	0	2	4	0	5	0	0	0
	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0
	N	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0
	P	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Q	0	0	0	0	0	0	0	0	0	0	0	0	0	5	1	0	0	2	0	0
	R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	9
	S	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	T	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	V	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	W	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	X	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Repulsives: 4 Attractives: 32 H-bonds(?): 98

TABLE 4
Frequency of g:e' Interactions in Type II Keratin Homodimers

eg-pair:		A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
5	A	0	0	0	5	0	0	0	0	1	2	0	0	0	5	5	0	0	1	0	0
	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
	D	0	0	0	0	0	0	0	0	0	2	0	0	0	0	5	0	0	1	0	0
	E	1	0	0	0	0	0	0	3	8	9	5	1	0	0	7	5	6	1	0	0
	F	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	G	0	0	0	0	0	0	0	0	0	2	1	0	0	1	1	0	0	0	0	0
	H	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
	I	0	0	0	0	0	0	0	0	3	0	0	0	0	4	8	0	1	1	0	0
	K	0	0	0	0	0	0	0	0	0	4	2	4	0	4	0	2	6	1	0	1
	L	0	0	0	0	0	0	0	0	0	0	0	1	1	1	11	3	5	0	0	0
	M	0	0	0	0	0	0	0	0	0	0	0	4	0	2	0	0	0	0	0	1
	N	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	5	2	0	0
	P	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Q	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	4	0	0	2
	R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	1	0	1
	S	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	T	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0
	V	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	W	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	X	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Y	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Repulsives: 1 Attractives: 20 H-bonds(?): 81

TABLE 5
Frequency of g:e' Interactions in Keratin Heterodimers

eg-pair:

	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
A	1	0	3	2	0	0	0	5	7	2	0	1	0	11	0	0	0	0	0	0
C		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
D			1	2	0	0	0	5	6	3	0	1	0	0	4	0	6	0	0	0
E				1	0	4	0	2	29	13	4	8	0	11	17	6	4	2	0	1
F					0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
G						0	0	0	1	5	3	0	0	1	2	0	0	2	0	0
H							0	0	0	1	0	0	0	0	0	0	0	0	0	0
I								0	0	0	0	2	0	1	15	0	1	0	0	1
K									2	12	4	5	0	7	1	1	4	3	0	0
L										0	2	5	1	8	10	3	10	1	0	0
M											0	0	0	7	1	0	1	0	0	0
N												0	0	9	1	0	6	0	0	0
P													0	0	0	0	0	0	0	0
Q														4	2	1	7	1	0	2
R															0	1	6	3	0	10
S																0	3	0	0	0
T																	0	0	0	0
V																		0	0	4
W																			0	0
Y																				0

Repulsives: 7 Attractives: 56 H-bonds(?): 186

Paradoxically, oppositely charged residues occurred at g and succeeding e' positions with approximately equal frequencies in each keratin heterodimer and the homodimers of its constituent chains. Table 5 showed the presence of 56 attractive pairs in the heterodimers. This number was similar to the total number (*i.e.*, 20 and 32) of repulsive pairs observed in the constituent homodimers (Tables 3 and 4). The individual pairs of chains showed the same trend. The number of repulsive g-e pairs was also similar in the homodimers and heterodimers. This pattern was at odds with experiments showing that complementary charges at g and succeeding e' positions in heterodimers, coupled with repulsive g-e' charge pairs in the constituent homodimers, could favor heterotypic pairing. Consequently, the results demonstrated that the keratins achieve heterospecificity primarily by core pairing and by changes in the context of g-e' ion pairs.

The pairing preference data for a:a' amino acids and d:d' amino acids shown in Table 2, in combination with the known principle of repulsive interactions relieved by charge complementation at g and succeeding e' positions was used to design and synthesize a polypeptide probe capable of heterospecific binding to a target polypeptide sequence.

EXAMPLE 2

Design And Synthesis Of A Polypeptide Probes (anti-APCp1 and anti-APCp2) Specific For APC

The coiled coil domain of the APC tumor suppressor protein was chosen as a target for the peptide probe design. Mutations in the APC gene are associated with 75% of inherited and sporadic colon tumors (Nakamura, *J. Cancer Res. Clinical Onco.* 121, 529-34 (1995)). Additionally, APC is known to bind and down regulates β -catenin, suggesting a role in regulation of cell adhesion. (Munemitsu *et al.*, *Proc. Natl. Acad. Sci. USA* 92, 3046-3050 (1995); Rubinfeld *et al.*, *Science* 262, 1741-43 (1993); Polakis, *Curr. Op. Genetics and Development* 5, 66-71 (1995)). Disease-associated APC alleles usually encode truncated, prematurely terminated proteins. (Joslyn *et al.*, *Proc. Natl. Acad. Sci. USA* 90, 11109-11113 (1993); Groden *et al.*,

Cancer Res. 55, 1531-9 (1995); Polakis (1995)), therefore the tumorigenic proteins contain the coiled-coil domain targeted by our anti-APC peptide. Groden (1991); Joslyn (1993).

Most importantly, the amino-terminal 55 amino acids of the APC protein contain a coiled coil motif and mediate homodimerization of APC fragments as a parallel coiled coil. Joslyn (1993). Since this 55 amino acid region is present in all the truncated forms of the APC protein, both the wild-type form and all the truncated transforming alleles of APC contain the coiled coil domain that is the target of the probe.

A. Principles of Designing A Polypeptide Probe Specific For the Coiled Coil Region of APC

The design of the polypeptide probe specific for APC was based on the amino acid sequence (SEQ ID NO:) (GenBank Accession No. M73548) of amino acids 2-55 of wild type APC (APC-55). The APC-55 sequence was specified by expression of a nucleic acid fragment encoding this sequence, and the molecular mass of the polypeptide expression product was verified as follows. APC-55, a peptide corresponding to amino acids 2-55 of human APC, was produced by expression in *E. coli* (Joslyn *et al.*, 1993). The gene fragment encoding this sequence was inserted between the NdeI and BamHI sites in the pAED4 plasmid, and BL21 (DE3) cells were transformed by the CaCl₂ method. Fresh transformants were grown in LB medium to OD₆₀₀ = 0.8 and induced with 1 mM IPTG. Cells were harvested after five hours and stored at -70° C. Thawed cell pellets were resuspended in 80 mM TrisHCl pH8.0, 0.2 mM EDTA, 20 mM KCl and lysed by sonication in a dry ice/ethanol bath. The pH was lowered to 2 by dropwise addition of concentrated HCl, and the acidified lysate was cleared by centrifugation.

The supernatant was neutralized, diluted two-fold and loaded onto DEAE cellulose. The column was developed with 10-1000 mM KCl. The peak fractions of peptide were concentrated and purified by reverse phase HPLC on a C18 semiprep column (Vydac). A 0.2%/min gradient of 0.1% TFA eluted a single peak. The peak fractions were lyophilized and resuspended in water. The amino acid composition of

the peptide was confirmed by electrospray mass spectrometry. The sequence of APC-55 was determined to be

MAAASYDQLLKQVEALKMENSNLROELEDNSNHLTKLETEASNMKEVLKQL
QGS (SEQ ID NO:). The underlined residues correspond to amino acids that were
targeted for change in the design of the anti-APC peptide.

As explained in the Detailed Description above, two general principles were used to introduce specificity into the APC peptide probe. The first guiding principle was that core residues were chosen to conserve packing volume and to introduce **a:a'** and **d:d'** pairs that occur at enhanced frequency in the sequences of keratin heterodimers as shown in Table 2. The second guiding principle was that charged residues were placed at e and g positions in the probe to form favorable electrostatic interactions with apposed g' and e' residues in the target and, at the same time, to form repulsive interactions with apposed g' and e' residues in the probe. Thus, destabilizing interactions were introduced into the probe that could be relieved in the heterodimer.

In addition to these two guiding principles, additional changes at the f positions were made to change the overall charge of the probe to facilitate experimental analysis of pairing, to preserve the overall stability of the system using residues with significant helical propensity, to promote the solubility of the probe peptide, and to introduce chromogenic groups to allow the probe to be detected optically. These changes were not expected to contribute to the preference of the anti-APC peptide for its target. Based on the above, altogether 20 changes from the wild type APC sequence were made to generate anti-APCp1 and anti-APCp2 peptides.

TABLE 6		
Design Rationale for Sequence-Specific anti-APC		
Residue	Change	Rationale
Met1		Absent in purified APC-55 due to cleavage <i>in vivo</i>
Ala2	Met/Nr1	Complement core packing defect*
Ser5	Lys	Destabilize anti-APC homodimer (+/+ with Lys10)
Tyr6	Gly	Complement core packing defect*

Leu10	Lys	Destabilize anti-APC homodimer (+/+ with Lys5)
Gln12	Glu	Stabilize Heterodimer (+/- Lys17) Destabilize anti-APC homodimer (-/- with Glu17)
Lys17	Glu	Destabilize anti-APC homodimer (-/- with Glu12)
Met18	Tyr	Chromophore
Gln25	Lys	Increase helical propensity and net charge
Glu26	Lys	Destabilize anti-APC homodimer (+/+ with Lys31)
Asn30	His	Complement core packing defect*
Ser31	Lys	Stabilize heterodimer (+/- with Glu26) Destabilize anti-APC homodimer (+/+ with Lys26)
Asn32	Lys	Increase helical propensity and net charge
His33	Lys	Stabilize heterodimer (+/- with Glu38) Destabilize anti-APC homodimer (+/+ with Lys38)
Glu38	Lys	Destabilize anti-APC homodimer (+/+ with Lys33)
Ala41	Ile	Complement core packing defect*
Met44	Ala	Complement core packing defect*
Glu46	Lys	Increase helical propensity and net charge
Val47	Met/Nrl	Heterospecificity (interacts with Gln52)*
Gln52	Tyr	Heterospecificity (interacts with Val47)*
Gly53	Ala	Increase helical propensity

* Changes based on analysis of keratin coiled-coil sequences.

Statistically preferred pairs were compared to the wild-type APC-55 sequence, and five changes were made to the a and d positions in the probe, Ala2Met/Nrl, Tyr6Gly, Asn30His, Ala41Ile and Met44Ala (Table 6). Four pairs of sequence changes were made to destabilize probe/probe oligomerization with repulsive charge/charge interactions Ser5Lys, Leu10Lys, Gln12Glu, Lys17Glu, Glu26Lys, Ser31Lys, His33Lys, and Glu38Lys. These unfavorable repulsive interactions were designed to be alleviated by heterodimerization with the APC peptide. Two

additional changes at the e and g positions were made in order to increase heterospecificity: Val47Met/Nrl and Gln52Tyr. Woolfson (1995).

Asn20, at an a position, remained in both peptides to facilitate an interhelical buried polar interaction often observed in coiled coils (Alber (1992)) and has been proposed to aid specificity in chain orientation and heptad alignment. Lumb, 1995. Gln25, Asn32, and Glu46 (all found in the f position) were changed to Lys in the probe in order to increase helical propensity (O'Neil *et al.*, *Science* 250, 646-651 (1990)) and the net charge of the probe for solubility. Gly53 (an f position) was also changed to Ala in order to increase helical propensity. Finally, Met18 (also an f position) was changed to a Tyr in order to add a chromophore to aid in concentration determination. As discussed above, the changes at the b, c and f positions were not expected to influence the specificity (i.e., pair preference) of the probe.

Based on the above rationale, the anti-APCp1 probe sequence was MAAKGDQLKKEVEALEYENSNLRKKLEDHKKKLTKLKTEISNAKKMLKQLY ASI (SEQ ID NO:). Changes from the wild type sequence are underlined. The first-designed anti-APC sequence (anti-APCp1) contained methionine residues at two positions. This peptide was specific for the N-terminal APC coiled coil, as judged by thermal denaturation experiments showing thermal stability at 20 μ M peptide as a function of concentration in the presence of equimolar amounts of APC (Table 7).

TABLE 7

Concentration Dependence on Thermal Stability

Peptide	Apparent T_m^* ($^{\circ}$ C)		
	8.0 μ M	20.0 μ M	75.0 μ M
APC-55	36.3	40.0	46.5
Anti-APCp1	45.0	52.3	59.6
APC-55/Anti-APCp1	50.6	54.3	64.7

* calculated from the midpoint of transition of thermal denaturation curves generated by CD spectroscopy at 222 nm. Samples are in 10mM phosphate pH7.0 and 100mM potassium fluoride.

However, the methionine side chain was found to be subject to oxidation. To block oxidation the methionines were replaced with the nonbiological, isosteric amino acid, norleucine to generate anti-APCp2

[NrlAAKGDQLKKEVEALEYENSNLRRKKLEDHKKKLTCLKTEISNAKKNNrlLKQ
LYASI (SEQ ID NO:); the underlined amino acid is norleucine].

Anti-APCp1 and anti-APCp2 were identical except that Met2 and Met47 in anti-APCp1 were replaced by the nonbiological, isosteric Nrl residue in anti-APCp2. These changes were made to eliminate the possibility of oxidation of the methionines at positions 2 and 47. The anti-APCp1 is capable of being produced *in vivo*, while anti-APCp2 can be produced only by chemical synthesis.

Figure 2 provides a Helical wheel diagram showing the sequences of the target APC-55 peptide (left) and the designed anti-APCp2 probe (right). The residues in the wild-type APC-55 peptide are shown in outlined letters, and the sequence changes in anti-APCp2 are shown in bold. The sequences begin with Ala2 (APC-55) and Nrl2 (anti-APCp2) are position **d**. The view is from amino-terminus along the superhelical axis. The **a** and **d** residues form alternating layers, and potential interhelical ion pairs between **g** and succeeding **e'** residues are marked with arrows. As explained above, of the 20 changes in anti-APCp2, five were made based on covariation frequencies (Table 2) and eight changes at the **e** and **g** positions were made to introduce repulsive ionic interactions in the anti-APC homodimer that are relieved in the parallel heterodimer. Two additional changes at the **e** and **g** positions and five changes at the non-interacting **f** position were made to add chromophores or to increase the helical propensity or the net charge of the designed peptide.

The anti-APCp2 was also demonstrated to be specific for the N-terminal APC coiled coil, as judged by the absence of concentration dependent thermal stability of the hetero-oligomer of anti-APC-p2 and APC-55 (Table 8).

TABLE 8

Thermal stability at 20 μ M peptide concentration in PBS (pH 7.0)					
Peptide	APC-55	anti-APCp1	anti-APCp2	APC + anti-APCp	APC + anti-APCp2
T _m (°C)	40.0	52.3	58.2	54.3	57.5

As noted above, Figure 2 shows the amino acids at each of the **a, b, c, d, e, f,** and **g** positions of APC-55 (open letters) and the amino acids at each of the **a', b', c', d', e', f',** and **g'** positions of the designed anti-APCp2. The replacement of methionine with norleucine caused the denaturation temperature of the anti-APC peptide to rise, but the anti-APCp2 peptide still showed specificity for APC (Table 8). These results demonstrate that the nonbiological amino acid, norleucine, was not necessary to achieve the preference of the probe for APC.

B. Peptide Synthesis And Purification

Following the design of the anti-APCp1, the anti-APCp1 and anti-APCp2 polypeptides as well as the APC-55 polypeptide were synthesized with F-moc chemistry using an Applied Biosystems 581A peptide synthesizer. As noted above, methionines in anti-APCp1 were replaced with isosteric norleucine (Nrl) in anti-APCp2 to prevent oxidation of the side chain. Peptides were cleaved from the resin with hydrofluoric acid (HF), lyophilized, resuspended in water and purified by reverse phase HPLC using the same column and gradient as for the wild-type sequence. The amino acid composition and purity of the peptides were checked by HPLC analysis and electrospray mass spectrometry.

EXAMPLE 3

The anti-APCp1 And Anti-APCp2 Polypeptide Probes Bind Specifically to APC And Preferentially Form Hetero-oligomers

To determine whether the anti-APCp1 and anti-APCp2 polypeptide probes bind specifically to APC and whether they form hetero-oligomers with APC in

preference to homo-oligomers, the interactions between the probes and APC were examined using (a) circular dichroism spectroscopy, (b) equilibrium ultracentrifugation, and (c) native gel electrophoresis as follows.

A. Circular Dichroism Spectroscopy

CD spectra (180 - 300 nm) for the APC-55 and anti-APCp1 peptides alone and in combination were recorded in a 1 mm quartz cuvette at 5° C using an Aviv 62DS circular dichroism spectrophotometer. The concentrations of peptide stocks were determined optically by the method of Gill and vonHippel, *Anal. Biochem* 182:319-326 (1989). Peptide stocks were diluted in 10 mM KPO₄, 100 mM KF, pH 7.0. Thermal denaturations were carried out in steps of 1-3° C with 2 minutes for equilibration and one minute for data averaging. Thermal transitions were >95% reversible. The data are shown in Figures 3 and 4. The results in Figures 3 and 4 obtained with the APC-55 alone are shown by open circles (○), with the anti-APCp1 alone by squares (□), and with an equimolar mixture of APC-55 and anti-APCp1 with closed circles (●).

The results demonstrate that the designed peptide probe, and an equimolar mixture of the probe and the wild-type APC-55 peptide were at least 95% helical at 75μM. Thermal denaturation experiments followed by circular dichroism at 222nm demonstrated an increase greater than 10°C in the thermal melting transition when the APC-55 peptide and the probe were mixed in equal amounts.

The stability of the hetero-oligomerization was determined by the investigation of the dependence of apparent T_m on peptide concentration. These results are shown in Table 9.

TABLE 9

Dependence of apparent T_m on peptide concentration.			
Peptide	Apparent T_m (+/- 1.25 °C)		
	8.0 μM	20.0 μM	75.0 μM
APC-55	36.3	40.0	46.5

anti-APCp2	51.5	58.6	66.4
APC-55/anti-APCp2	52.9	57.5	63.6

The higher thermal stability of the heteromeric mixture was consistently observed at different peptide concentrations (Table 9). The observed increase in T_m with increasing peptide concentration is consistent with the formation of oligomers.

B. Equilibrium ultracentrifugation.

The APC-55 and anti-APCp1 peptides alone and in combination were analyzed by equilibrium centrifugation using a Beckman X-LA ultracentrifuge. Samples were analyzed at three total peptide concentrations (0.075, 0.150, and 0.250 mM) in 10 mM KPO₄, 100 mM KF, pH 7.0. Absorbance was monitored at 215, 230 and 280 nm. Solvent density was assumed to be 1.0005 g/l at 278° K. Partial specific volumes were calculated from the amino acid compositions of the peptides. Apparent molecular weights were calculated by simultaneously fitting all data sets for one species to a single molecular weight with the program HID4000. The results of the equilibrium ultracentrifugation are shown in Table 10.

TABLE 10

Molecular weights determined by ultracentrifugation		
Peptide	calculated MW	observed MW
APC-55	12,200	12,400
anti-APCp2	12,500	14,000*
APC-55/anti-APCp2	12,300	13,300

* Systematic residuals indicate multiple oligomeric species

The results shown in Table 10 confirmed the heteromeric mixture to be a dimer, consistent with the dimeric coiled coil design (Table 6). Importantly, the probe by itself did not form a single oligomeric species in solution as shown by the observed molecular weight of 14,000 (Table 10). This demonstrated that the anti-APCp2 polypeptide forms homo-oligomers other than and in addition to a homodimer. The

additional observation that the anti-APCp2 polypeptide, which forms homodimers as well as homotrimers, and possibly homotetramers, was capable of hetero-dimerization with APC-55 demonstrates that a coiled coil polypeptide probe containing more than two polypeptide strands is capable of heterospecific oligomerization with a target coiled coil polypeptide.

C. Native gel electrophoresis

Native acrylamide gel electrophoresis was used as a direct test of heterodimer specificity. Samples of the individual peptides and an equimolar mixture of the peptides were prepared at 0.6 mM and diluted two-fold in 0.2% (w/v) methyl green, 20% glycerol, 750 mM beta-alanine acetate pH 6.0. Gels contained a separating gel of 7.5% acrylamide in 375 mM beta-alanine acetate, pH 4.0. Samples were run for 2.5 hours at 100 volts, and the gels were fixed with 2% glutaraldehyde and stained for at least 1 hour in 0.2% Coomassie Brilliant Blue in 50% methanol, 10% acetic acid (v/v). Destaining was carried out overnight in the same solvent lacking the dye.

The results of the native gel electrophoresis are shown in Figure 5. Figure 5C shows that when the heterodimer (an equimolar mixture of the two peptides) was used, a new single species with an intermediate mobility was detected. The new species had a mobility consistent with the calculated isoelectric point of the heterodimer (pI=10.0). The appearance of the heterodimeric species with concomitant loss of the homo-oligomers demonstrates the high specificity of the probe for the wild-type APC-55 peptide. This data also demonstrate that the preference for the formation of a heterodimer was at least 100 fold greater than the preference for the formation of homodimers as judged by the sensitivity of the detection method on native gels. The data presented herein was surprising in view of the prior art reports of the sufficiency of e':g interactions for dimerization specificity of coiled coil proteins [O'Shea *et al.* (1992), *supra*; O'Shea *et al.* (1993) *supra*; Krylov *et al* (1994) EMBO J. 13:2849-2861].

Further experiments to quantitate the level of specificity for heterodimerization of the anti-APCp2 probe with APC using fluorescence energy transfer titration by measuring the change in fluorescence between APC and buffer

titrated with anti-APCp2 did not yield a numerical value for the heterodimerization specificity as the detection threshold of this method was greater than that necessary for such determination. Based on the sensitivity of the fluorescence method used, the dissociation constant of the APC-55/Anti-APCp2 heterodimer was estimated to be less than 10 nM. This high affinity demonstrates that the anti-APCp2 probe is useful at pharmacological concentrations.

EXAMPLE 4

The anti-APC Polypeptide Probe Is Heterospecific For APC in Bacterial Cell Lysates

In order to test for dimerization specificity of the anti-APCp1 for APC fragments in the presence of many other cellular proteins, APC protein fragments were expressed in *E. coli* as fusion proteins with glutathione (GST). The specificity of binding of anti-APCp2 to APC was compared to the specificity of binding of a monoclonal anti-APC antibody on Western blots as follows. Cell extracts containing the fusion proteins were separated by SDS-PAGE and blotted. Biotinylated anti-APCp2 was used to detect these fusion proteins by binding the probe to the blotted APC fusion proteins and then developing the blot with streptavidin-HRP. Phosphorescence was detected on X-ray film. These steps are described more fully in the following sections.

A. Expression of APC Fusion Proteins

In frame, C-terminal fusions between APC sequences and glutathione S-transferase (GST) were constructed in the expression vector pGEX-2TK (Pharmacia). Enzymes for cloning were obtained from Life Technologies. The relevant sequence of each plasmid was confirmed using an Applied Biosystems 373A automated DNA sequencer.

i. pGEX-2TK/APC-H1

DNA encoding the first 55 amino acids of the human APC protein (heptad repeat 1) was PCR-amplified from a full length *APC* cDNA using Taq DNA

polymerase and tailed primers that generated an EcoR1 cassette. (PCR primers:
Forward - 5'-TGTAACGACGGCCAGTGGGAATTCATATGGCTGCAGCTT
CAGTTG-3' (SEQ ID NO:); Reverse: 5' - CAGGAAACAGCTATGACCGA
ATTCCTAAATACTTCCTTGTAGTTGTTTA - 3' (SEQ ID NO:)). The product and
5 the vector were digested with EcoR1 and ligated.

ii. pGEX-2TK/APC-H1-4

An NdeI-SmaI DNA cassette encoding the N-terminal 373 amino acids of the
APC protein (heptad repeats 1-4) was generated by restriction digestion of an APC
10 cDNA construct in pBluescript. Following purification of the fragment by agarose gel
electrophoresis, the NdeI overhang was filled in with Klenow DNA polymerase, and
the resulting blunt-ended cassette was ligated into pGEX-2TK linearized with SmaI.

iii. pGEX-2TK/APC-PROBE

15 A BamH1-EcoR1 cassette encoding the anti-APCp1 probe peptide was created
by synthesizing oligonucleotides to the 5' (probe5) and 3' (probe3) halves of anti-
APCp1 gene sequence. The probe5 oligonucleotide was PCR-amplified using tailed
primers to generate a double-stranded, BamH1-AvaII cassette, and the probe3
oligonucleotide was PCR-amplified with tailed primers to produce an AvaII-EcoR1
20 cassette. The probe 5 and probe3 cassettes were digested with AvaII, ligated and
purified on a 6% polyacrylamide gel. The resulting anti-APCp1 gene and the vector
were digested with BamH1 and EcoR1 and ligated.

iv. Bacterial cell lysates.

Whole cell lysates were obtained by the method of Smith and Johnson (1988) .
25 from *E. coli* BL21 (DE3) expressing each of the GST fusion proteins. Bacteria
carrying each pGEX-2TK-derived plasmid were cultured overnight at 37° C in L
broth supplemented with 50 mg/l ampicillin (Boehringer Mannheim). Overnight
cultures were diluted 1:10 in fresh medium and grown an additional hour at 37° C.
Expression of GST fusion proteins was induced by adding IPTG (ICN Biochemical)
30 to a concentration of 0.1 mM. Induced cells were grown 3 hours at 37° C, harvested

by centrifugation at 4° C, and washed in 10 ml PBS buffer (20 mM NaPO₄, 150 mM NaCl, pH 7.3). Cells were lysed by sonicating for 3 x 30 seconds in PBS containing 0.1 % Triton X-100 (United States Biochemical), and the lysates were clarified by centrifugation. The supernatants containing GST alone and the GST-APC-H1 polypeptides were adjusted to a total protein concentration of 1 mg/ml in SDS PAGE sample buffer (62.5 mM TrisHCl, pH 6.8, 10% glycerol, 2% SDS, 0.7 mM 2-mercaptoethanol, 0.00125% bromophenol blue) and used for Western blotting analysis. The GST-APC-H1-4 and GST-APC-probe polypeptides were insoluble. The pellets from the lysates containing them were resuspended in PBS and adjusted to 1 mg/ml protein concentration in SDS PAGE sample buffer.

B. Western Blot Analysis

Bacterial lysates containing GST, GST-APC-H1, GST-APC-H1-4 and GST-APC-probe were diluted 1:10 in cell lysates from induced BL21 cells containing no APC sequences and adjusted to a final protein concentration of 0.5 mg/ml in sample buffer. Five micrograms of each sample was electrophoresed on a precast 10% Tricine-SDS polyacrylamide gel (Novex) as described by Schager and vonJagow (1987). Proteins were transferred to 0.45 micron nitrocellulose for 2 hours at 100 V, 4° C, in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS).

i. Detection using anti-APCp1 Sequences.

The anti-APCp1 peptide was biotinylated by removing the F-moc protecting group from the N-terminus of the peptide on the synthesis resin and incubating the resin with the N-hydroxy-succinimidyl ester of biotin containing a long spacer arm (Pierce, Catalog #21336) in dimethylformamide. The biotinylated anti-APCp1 contains Met (instead of Nrl) as well as an additional Leu8Val mutation and Glu-Asp-Glu (wild-type APC sequence) at the peptide C-terminus. This probe was indistinguishable from anti-APCp1 in native gel experiments and CD experiments (data not shown). Blots were blocked for 1 hour in PNT buffer (10mM phosphate, pH 8.0, 175 mM NaCl, 0.1% Tween-20) containing 0.5% bovine serum albumin. After blocking, blots were probed for 1 hour at 42° C with 5 nM biotinylated antiAPC

peptide in PNT buffer containing 1% BSA. After incubation for 30 minutes at room temperature in streptavidin-horseradish-peroxidase diluted 1:2000 in PNT plus 1% BSA, the blots were washed 3 times for 15 minutes in PNT buffer and developed using ECL substrate (Amersham).

5 ii. **APC detection using APC Ab-1.**

Blots were blocked for 1 hour in TBST (10 mM phosphate, 175 mM NaCl, pH 8.0, 0.1% Tween-20) containing 5% BSA. After blocking, blots were probed with mouse monoclonal APC antibody Ab-1 (Oncogene Science/Calbiochem), which recognizes the N-terminal 55 amino acids of the APC protein (Smith KJ, *et al.*, (1993) *Proc. Natl. Acad. Sci USA* 90, 2846-2850). The antibody was diluted 1:3000 in TBST, 1% BSA. Blots were washed 3 times for 5 minutes each in TBST and incubated for 1 hour in horseradish peroxidase-conjugated rabbit anti-mouse IgG₁ antibody diluted 1:30000 in TBST, 1% BSA. After three 15-minute washes in TBST, blots were developed using ECL substrate (Amersham).

15 The results of the Western blot analysis are shown in Figure 6. The SDS-PAGE prepared as described above is shown in Figure 6C. This SDS-PAGE was blotted and the blots were developed with either biotinylated anti-APC monoclonal antibody (Figure 6A) or biotinylated anti-APCp1 polypeptide probe (Figure 6B). The results in Figure 6 demonstrate that anti-APCp1 peptide can detect APC fragments with specificity even when all other *E. coli* proteins were present in the probed cell extract, thus demonstrating the specificity of the anti-APCp1 probe for APC.

20 Further, anti-APCp1 and antibodies against the amino-terminus of APC detected similar patterns of GST-APC fusion proteins expressed in *E. coli* (Fig. 6). In particular, Western blots developed with the antisense peptide and the antibody revealed common regions of proteolytic susceptibility within the first 373 amino acids of APC. In contrast to the antibody, the anti-APCp1 peptide also bound to a GST fusion with the antisense sequence itself (Fig. 6B, lane 4). Thus, the twenty sequence changes in anti-APCp1 apparently abolished the antibody epitope but preserved the capacity for homo-oligomerization. These results suggest not only that the antisense

peptide forms a coiled-coil interaction with the APC amino terminus, but also that this coiled coil forms in preference to interactions with *E. coli* proteins.

EXAMPLE 5A

The anti-APC Polypeptide Probe Is Sensitive To Low Endogenous *in vivo* Cellular Concentrations of Human APC

Precipitation experiments were carried out in order to determine whether the anti-APCp1 polypeptide probe was capable of detecting the low *in vivo* levels of APC which are typically present in normal and tumor cells. Figure 7 is a schematic representation of the strategy used. Briefly, cell lines which produce a normal (i.e., full length human APC) and mutant (i.e., truncated human APC) were lysed, and the lysate combined with either biotinylated anti-APCp2 or biotinylated anti-APC monoclonal antibody. Molecules bound to the biotinylated probes were collected by binding to streptavidin, and the bound molecules released from the beads followed by SDS-PAGE, blotting of the gels, and detection with a biotinylated APC antibody. Phosphorescence was detected on X-ray film. These steps are elaborated in more detail as follows.

A. Cell Lines

1. DLD-1 (ATCC CCL-221):

The DLD-1 cell line expresses an approximately 150 kDa truncated mutant APC in the absences of full-length APC expression. These cells were cultured in RPMI1640 medium (Gibco BRL) supplemented with 10% fetal calf serum (HyClone Laboratories).

2. SW480 (ATCC CCL-228):

The SW480 cell line expresses an approximately 150 kDa truncated mutant APC. While it is generally accepted in the art that SW480 does not express full-length APC protein, faint bands were observed migrating at the size of full-length APC that cross-react with APC antibodies in SW480 Westerns and

immunoprecipitations. SW480 cells were cultured in Leibowitz's L-15 medium (Gibco BRL) supplemented with 10% fetal calf serum.

3. LS174T (ATCC CL-188):

5 The LS174T cell line expresses full-length APC protein. There are no known APC mutations in this cell line. LS174T is thought to be derived from a tumor that arose via the "genetic instability" pathway rather than the APC pathway. This cell line was cultured in Minimal Essential Medium (Gibco BRL) supplemented with 10% fetal calf serum and 1% non-essential amino acids.

B. Probe Precipitation Protocol

10 1. Lysate preparation

Cells were harvested at 90-100% confluency. Cells were washed twice with ice-cold Dulbecco's phosphate buffered saline (Gibco BRL) containing magnesium chloride and calcium chloride, scraped into 15 mL ice cold D-PBS and pelleted in a Beckman J-6 centrifuge for 5 minutes at 4°C. The cell pellet was resuspended in 15 mL ice-cold phosphate buffer, pH 8, containing 0.2% NP-40, 400 µg/mL of Pefabloc SC (Boehringer Mannheim) and 10 µg/mL each of leupeptin, pepstatin, and aprotinin. The suspension was sonicated 3 x 30s at 4° C, then pelleted for 15 minutes at 4°C in a microcentrifuge. This supernatant, which should contain both nuclear and cytosolic proteins was used for APC precipitation.

20 2. Precipitation

2 nmol biotinylated probe (B-Probe), unbiotinylated probe (Probe) or D-biotin were added to aliquots of cell lysate (aliquots typically contained 1.5-2 mg total protein by Bradford assay) and incubated with constant rocking for one hour at 4° C. After one hour, 2 mg streptavidin magnetic particles (Boehringer Mannheim) were added to each reaction and incubated, rocking, for one hour at 4°C. Streptavidin magnetic particles were preblocked overnight in lysis buffer containing 2.5% bovine serum and 2.5% casamino acids (Difco). Pellets were washed five times in lysis buffer containing protease inhibitors, and the final supernatant was removed.

Subsequently, pellets were incubated for 10 minutes at 95° C in 20 μ L lysis buffer containing 1 mM D-biotin. After 10 minutes, 10 μ L SDS-PAGE sample buffer was added to each pellet and reactions were incubated an additional 5 minutes at 95° C. Precipitates were electrophoresed on 6% precast tris-glycine polyacrylamide gel (Novex) electroblotted to nitrocellulose and probed with APC Ab-1 (Oncogene Science) (Smith KJ, *et al. supra*, (1993)) as previously described.

C. Immunoprecipitation of APC protein using anti-APC antibodies

1. Lysate Preparation

Lysates were prepared exactly as described above except that cells were lysed in ice-cold Dulbecco's PBS supplemented with 100 mM KCl, 0.2% NP-40 and protease inhibitors.

2. Immunoprecipitation

Immunoprecipitation of APC was performed essentially as described above. 2.5 μ g each of anti-APC Ab-5 and Ab-6 (Oncogene Science) (Smith KJ, *et al.* (1993) *Proc. Natl. Acad. Sci USA* 90, 2846-2850) was added to each lysate aliquot; 2.5 μ g of nonspecific mouse IgG1 and IgG2b antibodies were added to separate aliquots as negative controls for the precipitation. Lysates were incubated with antibody for one hour at 4° C, rocking, then 50 μ L Protein G Agarose was added to the lysate and allowed to incubate for one hour. (Protein G agarose, 20%w/v, was preblocked overnight with 5% BSA in lysis buffer.) Following five washes with ice-cold lysis buffer, Protein G agarose pellets were resuspended in 15 μ L SDS PAGE sample buffer, incubated for 5 minutes at 95°C, then loaded on a 6% precast tris-glycine polyacrylamide gel. Western Blot analysis was performed as described above.

Figure 8 shows the results of the immunoprecipitation of full-length (two lower blots) and mutant truncated APC-55 forms (two upper blots) from human colon cell lines using either anti-APC antibody or the biotinylated anti-APCp2 polypeptide probe. This data demonstrates the specific binding of anti-APCp2 peptide to both wild-type (320 kDa) and mutant (200 kDa) APC proteins at very low endogenous cell concentrations. Furthermore, this data also demonstrates that the biotinylated

monoclonal antibody immunoprecipitations showed a similar band pattern as the designed coiled coil polypeptide probe.

EXAMPLE 5B

An additional affinity purification protocol was performed with Western blots of precipitations of APC from three different human colon cancer cell lines: 293 (Figure 9A), which expresses full-length APC, DLD-1 (Figure 9B), which contains a stop at codon 1417 and expresses a truncated APC molecule of 190 kDa, and HT-29 (Figure 9C), which expresses two truncated APC molecules of approximately 100 and 200 kDa (Morin *et al.*, *Proc. Natl Acad. Sci. USA* 93: 7950-54 (1996)).

Briefly, cells were harvested at 90-100% confluency, washed twice with ice-cold Dulbecco's phosphate buffered saline (D-PBS, Gibco BRL) containing $MgCl_2$ and $CaCl_2$, scraped into 15 ml ice cold D-PBS and pelleted in a Beckman J-6 centrifuge for 5 minutes at 4°C. Cell pellets were resuspended in 1 ml ice-cold phosphate buffer, pH8, containing 0.1% NP-40, 40 µg/ml of Prefabloc SC (Boehringer Mannheim) and 10 µg/ml each of leupeptin, pepstatin and aprotinin.

The suspension was sonicated, then pelleted for 15 minutes at 4°C in a microcentrifuge. For antibody-mediated precipitations 3 µg of anti-APC Ab1 (Oncogene Science/Calbiochem) or a non-specific mouse IgG₁ antibody (Sigma) was added. After 30 min on ice, 50 µL protein-G agarose (preblocked overnight with 5% BSA in lysis buffer) was added to the lysates and allowed to incubate for one hour, rocking at 4°C. Following five washes with ice-cold lysis buffer, protein G agarose pellets were resuspended in 15 µL SDS PAGE sample buffer and incubated for 5 minutes at 95°C.

For peptide-mediated precipitations, 2 nanomoles biotinylated-anti-APCp1, unbiotinylated anti-APCp1, or D-biotin were added on ice to aliquots of cell lysate (aliquots typically contain 0.5-1 mg total protein by Bradford assay). One milligram streptavidin magnetic particles (Boehringer Mannheim), preblocked overnight in lysis buffer containing 5% BSA was added to each reaction and incubated, rocking, for one hour at 4°C. Pellets were washed five times in ice-cold lysis buffer and incubated for

10 minutes at 55°C in 10 µL PBS containing 0.1 mM D-biotin. SDS-PAGE sample buffer (10 µL) was added to each pellet and reactions were incubated 5 minutes at 95°C. Precipitates were electrophoresed on 6% tris-glycine polyacrylamide gel (Novex), electroblotted to nitrocellulose and probed.

5 Blots were blocked for 1 hour in TBST (10 mM phosphate, 175 mM NaCl, pH 8.0, 0.1% Tween-20) containing 5% BSA and probed with anti-APC antibody Ab-1 (Oncogene Science/Calbiochem), or a monoclonal anti β -catenin antibody (Transduction Laboratories). Anti-APC Ab-1 was diluted 1:150 in TBST, 1% BSA and anti- β -catenin antibody was diluted 1:2000. Blots were washed 3 times for 5
10 minutes each in TBST and incubated 1 hour in horseradish peroxidase-conjugated rabbit anti-mouse IgG₁ antibody (Pierce) diluted 1:30,000 in TBST, 1% BSA. After three 15-minute washes in TBST, blots were developed using ECL substrate (Amersham). Western blots carried out with antibodies specific for the 20S
15 Laboratories) showed no staining of proteins associated with anti-APCp1 precipitations (data not shown), reflecting the specificity of the antisense peptide for the APC protein.

 As shown in Figure 9, blots were probed with an APC antibody (left panels) or a β -catenin antibody (right panels). The total lysate is shown in lane 1. Precipitations
20 were performed using: lane 2, biotinylated-anti-APCp1; lane 3, anti-APCp1 without biotin; lane 4, biotin alone; lane 5, the anti-APC antibody Ab-1; or lane 6, a non-specific mouse IgG. The biotinylated-anti-APCp1 precipitated truncation mutants and full-length APC at endogenous cell concentrations, demonstrating the affinity and specificity of the designed probe. Further, similar patterns and yields of
25 APC polypeptides were captured with biotinylated anti-APCp1 or a biotinylated monoclonal antibody specific for the APC amino terminus (Fig. 9A).

 Anti-APCp1 also coprecipitated β -catenin, a protein that recognizes motifs in the central third of the APC sequence (right panels) (Munemitsu (1995); Rubinfeld (1993)). These results suggest that the antisense peptide binds to APC at low
30 endogenous concentrations and pairs with full-length APC polypeptide chains without disrupting APC/ β -catenin complexes. Only a subset of β -catenin species

coprecipitated from tumor cell lines (Fig. 9), suggesting that anti-APCp1 is specific for APC and APC-associated proteins. Fluorescence energy transfer measurements using fluorescein- and coumarin-labeled anti-APCp1 and APC-55 (respectively) suggest an upper limit for the K_d of the heterodimer of 10 nM (data not shown).

5 Because the amino-terminal, coiled coil sequence targeted by anti-APCp1 occurs in tumor-associated truncation mutants, the distinct electrophoretic mobilities of the species detected by the antisense peptide distinguish wild-type from tumorigenic APC variants (Fig. 9).

10 In contrast to anti-APCp1, other protein-targeted ligands require experimental selections. Antibodies undergo selection and maturation *in vivo*, and iterative, system-specific binding assays are necessary to obtain protein ligands from phage display or chemical libraries (D. McGregor, *Mol. Biotechnol.* 6, 155-62 (1996); K. Janda, *Proc. Natl. Acad. Sci. USA* 91, 10779-85 (1994)). These experimental methods also necessitate purification of the target protein, and the chemical complexity of the
15 starting library limits the number of ligands screened. In contrast, the method used here to produce anti-APCp1 relies on simple, general pairing rules to design the sequence of a peptide capable of recognizing a target coiled coil.

Like complementary oligonucleotides, antisense coiled coils combine the utility of a sequence-based design and the simplicity of chemical synthesis. Unlike
20 antibodies, the antisense peptides are small, and they require no disulfide bonds for stability, no purification of the target protein and no use of animals. In addition, peptides directed against coiled coils necessarily disrupt the target structure (Goldman *et al.*, *J. Cell Biol.* 134, 971-983 (1996); Olive *et al.*, *J. Biol. Chem.* 271, 2040-2047 (1996); Olive *et al.*, *J. Biol. Chem.* 272, 18586-94 (1997); Sougen *et al.*, *Eur. J. Biochem.* 240, 765-773 (1996); Tripet *et al.*, *Prot. Eng.* 9, 1029-1042 (1996)). Coiled
25 coil sequences are readily identified by computer methods (Lupas (1996); Wolf (1997); Berger (1995)), making this large family of proteins amenable to antisense probe design. These distinctive properties suggest that antisense coiled coils may have broad uses *in vitro* and *in vivo*.

EXAMPLE 6

Sequence-Based Design of a Peptide Probe for Residues 6 to 49 of Yeast
Tropomyosin (TPM1)

Tropomyosin (TPM1) is essential in yeast for stabilizing actin cables and for
cytokinesis. The phenotypes of null mutants and the cellular distribution of
tropomyosin have been characterized. The sequence of the cloned gene shows that
tropomyosin contains many "violations" of the folding rules described *supra* in Tables
2-5, which can be compensated in a peptide probe designed in accordance with the
pairing rules of Table 2. The target sequence in TPM1 is a coiled coil sequence
KLSNLKLEAESWQEKYEELKEKNKDLEQENVEKENQIKSLTVK (SEQ ID
NO:). The rationale discussed in the following Table 11 was used to design a probe
specific for the TPM1 coiled coil target sequence.

TABLE 11

Sequence-based Design of a Peptide Probe for Residues 6 to 49 of Yeast Tropomyosin (TPM1)			
Design Rationale			
Position	Residue	Change	Rationale
g	Lys1	Glu	Stabilize heterodimer (+/- with Lys6) Destabilize Probe homodimer (-/- with Glu6)
e	Lys6	Glu	Stabilize heterodimer (+/- with Lys1) Destabilize Probe homodimer (-/- with Glu1)
g	Glu8	Arg	Heterospecificity (interacts with Gln 13) Destabilize Probe homodimer (+/+ with Lys13)
a	Ala9	Ile	Complement core packing*
b	Glu10	Lys	Increase helical propensity and net charge
c	Ser11	Lys	Increase helical propensity and net charge
d	Trp12	Leu	Complement core packing*

e	Gln13	Lys	Stabilize heterodimer (+/- with Glu8) Destabilize Probe homodimer (+/+ with Lys8)
g	Lys15	Glu	Stabilize heterodimer (+/- with Lys20) Destabilize Probe homodimer (-/- with Glu20)
a	Tyr16	Thr	Complement core packing*
b	Glu17	Lys	Increase helical propensity and net charge
	Glu18	Lys	Increase helical propensity and net charge
e	Lys20	Glu	Stabilize heterodimer (+/- with Lys15) Destabilize Probe homodimer (-/- with Glu15)
g	Glu29	Lys	Stabilize heterodimer (+/- with Glu34) Destabilize Probe homodimer (+/+ with Lys34)
a	Asn30	His	Complement core packing*
d	Lys33	Ala	Complement core packing*
e	Glu34	Lys	Stabilize heterodimer (+/- with Glu29) Destabilize Probe homodimer (+/+ with Lys29)
f	Asn35	Tyr	Chromophore for concentration determination
g	Gln36	Lys	Destabilize Probe homodimer (+/+ with Arg41)
e	Thr41	Arg	Heterospecificity (interacts with Gln36) Destabilize Probe homodimer (+/+ with Lys36)
f	Val42	Glu	Increase solubility

* Changes based on analysis of keratin coiled-coil sequences.

15 Based on the above rationale, the following probe is synthesized:

ELSNLEELRIKKLKEETKKLEEKNKDLEQKHVEAKYKIKSLREK (SEQ ID NO:).

Changes in the TPM1 sequence are underlined.

EXAMPLE 7

Sequence-based Design of a Peptide Probe For Residues 846 to 913
of *C. elegans* Dpy-27

Dpy-27 is a component of a complex of proteins required to globally reduce
the level of gene expression from the X chromosome in *C. elegans* hermaphrodites.
This protein complex localizes to the X-chromosome. The target coiled coil was
identified from the Dpy-27 sequence using the program PairCoil. This target Dpy-27
coiled coil had the sequence

RMVNYREVTVEDLDEKRAQIADLKR VQEE SQKSSAKIKQQIEQYKRKMFME
LVQK (SEQ ID NO:). The rationale discussed in the following Table 12 was used to
design a probe specific for the Dpy-27 target sequence.

TABLE 12

Sequence-based Design of a Peptide Probe for Residues 846 to 913 of <i>C. elegans</i> Dpy-27			
Design Rationale			
Position	Residue	Change	Rationale
g	Arg1	Glu	Stabilize heterodimer (+/- with Arg6) Destabilize Probe homodimer (-/- with Glu6)
a	Met2	Ile	Complement core packing*
d	Tyr5	Phe	Complement core packing*
e	Arg6	Glu	Stabilize heterodimer (+/- with Arg1) Destabilize Probe homodimer (-/- with Glu1)
g	Thr9	Arg	Stabilize heterodimer (+/- with Asp14) Destabilize Probe homodimer (+/+ with Arg14)
a	Val10	Ala	Complement core packing*
e	Asp14	Arg	Destabilize Probe homodimer (+/+ with Arg9)
f	Glu15	Tyr	Chromophore for concentration determination

g	Gln19	Glu	Stabilize heterodimer (+/- with Lys24) Destabilize Probe homodimer (-/- with Glu24)
e	Lys24	Glu	Destabilize Probe homodimer (-/- with Glu19)
g	Gln26	Arg	Destabilize Probe homodimer (+/+ with Arg31)
a	Val27	Ala	Complement core packing*
d	Ser30	Thr	Complement core packing*
e	Gln31	Arg	Destabilize Probe homodimer (+/+ with Arg26)
g	Ser33	Glu	Stabilize heterodimer (+/- with Lys38) Destabilize Probe homodimer (-/- with Glu38)
a	Ser34	Asn	Complement core packing*
d	Ile37	Ala	Complement core packing*
e	Lys38	Glu	Destabilize Probe homodimer (-/- with Glu33)
g	Gln40	Glu	Stabilize heterodimer (+/- with Lys45) Destabilize Probe homodimer (-/- with Glu45)
d	Tyr44	Phe	Complement core packing*
e	Lys45	Glu	Destabilize Probe homodimer (-/- with Glu40)
g	Lys47	Glu	Destabilize Probe homodimer (-/- with Glu52)
a	Met48	Leu	Complement core packing*
d	Met51	Ala	Complement core packing*
e	Phe52	Glu	Stabilize heterodimer (+/- with Lys47) Destabilize Probe homodimer (-/- with Glu47)
a	Leu55	Phe	Complement core packing*

* Changes based on analysis of keratin coiled-coil sequences.

Based on the above rationale, the following probe is synthesized:
EIVNFEEVEVRAYDLREKRAEIADLERRAEETRKENAKAEQEIEQFERELDRA
EMEFVQK (SEQ ID NO:). Changes in the DPY-27 sequence are underlined.

EXAMPLE 8**A Coiled Coil Polypeptide Probe Is A Useful Therapeutic Agent Against Infection With HIV Virus**

Of the anti-human immunodeficiency virus (HIV) treatment strategies tested to date, none is curative and only certain inhibitors of the viral reverse transcriptase (RT) and protease have demonstrated clinical benefit. As a result, the search for new, more efficacious drugs and/or other sites on the virus against which to target antiviral therapy continues at an urgent pace. The trimeric gp41 protein of HIV mediates fusion of the viral and host cell membranes. Trimerization of gp41 is mediated by helical regions comprising amino acids 540-590 and 624-666. Residues 540-590 are thought to form a central three-helical coiled coil onto which amino acids 624 associate (M. Lu, S.C. Blacklow and P.S. Kim, (1995), *Nature Structural Biology*, 2: 1075-1082).

This Example describes the generation of a coiled coil polypeptide capable of binding specifically to a coiled coil protein, the HIV gp41 protein, which is located on the surface of HIV and which may be useful as an antiretroviral agent. This Example involved (A) the design and synthesis of a coiled coil polypeptide which binds specifically to HIV gp41 protein, and (B) using the coiled coil polypeptide to inhibit *in vitro* viral activity.

A. Design And Synthesis Of A Coiled Coil Polypeptide Which Binds Specifically to HIV gp41 Protein

The target coiled coil identified in gp41 of HIV has the sequence:

QARQLLSGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARILAVEYLKDQQ
LLGIW (SEQ ID NO:).

The rationale discussed in the following Table 13 was used to design a probe specific for the gp41 sequence.

TABLE 13

Sequence-based Design of a Peptide Probe for
Residues 540 to 596 of *HIV* gp41

Design Rationale			
Position	Residue	Change	Rationale
d	Ala2	Leu	Complement core packing*, favor dimerization
a	Leu6	Met	Complement core packing*
d	Ile9	Ala	Complement core packing*, favor dimerization
g	Gln12	Glu	Destabilize Probe homodimer (-/- with Glu17)
a	Gln13	Val	Complement core packing*, favor dimerization
e	Leu17	Glu	Destabilize Probe homodimer (-/- with Glu 12)
g	Ala19	Glu	Destabilize Probe homodimer (-/- with Glu24)
a	Ile20	Val	Complement core packing*
d	Gln23	Met	Complement core packing*
e	Gln24	Glu	Destabilize Probe homodimer (-/- with Glu19)
g	Leu26	Thr	Favor dimerization**, improve solubility
a	Leu27	Ile	Complement core packing*
d	Thr30	Ser	Complement core packing*
e	Val31	Gln	Favor dimerization**, enhance solubility
f	Trp32	Lys	Enhance solubility
g	Gly33	Glu	Destabilize probe homodimer (-/- with Glu38)
a	Ile34	Val	Complement core packing*
d	Leu37	Ala	Complement core packing*
e	Gln38	Glu	Destabilize Probe homodimer (-/- with Glu33)
f	Ala39	Asn	Enhance solubility
a	Ile41	Val	Complement core packing*
d	Val44	Met	Complement core packing*
g	Tyr47	Glu	Destabilize Probe homodimer (-/- with Glu52)
a	Leu48	Val	Complement core packing*
d	Gln51	Met	Complement core packing*
e	Gln52	Glu	Destabilize Probe homodimer (-/- with Glu47)
f	Leu53	Asn	Enhance solubility
a	Gly55	Ile	Complement core packing*

Sequence-based Design of a Peptide Probe for Residues 540 to 596 of <i>HIV</i> gp41			
Design Rationale			
Position	Residue	Change	Rationale
d	Ala2	Leu	Complement core packing*, favor dimerization
a	Leu6	Met	Complement core packing*
b	Ile56	Glu	Enhance solubility
c	Trp57	Tyr	Enhance solubility, introduce chromophore

* Changes based on analysis of keratin coiled-coil sequences

** D.N. Woolfson and T. Alber (1995), *Protein Science*, 4: 1596-1607.

Based on the above rationale, the following anti-gp41 probe is synthesized:
 QLRQLMSGAVQEVNNLEREVEAMEHTIQLSOKEVKQAENRVLAMEREVKD
 MENLIEY (SEQ ID NO:). Changes in the GP41 sequence are underlined. This anti-
 gp41 coiled coil sequence is aimed at preventing assembly of the gp41 trimer by
 preferentially forming a heterodimer with residues 540-596. The specificity of the
 coiled coil polypeptide for the gp41 protein is confirmed using circular dichroism,
 equilibrium ultracentrifugation, and native acrylamide gel electrophoreses as described
supra in Example 3.

B. Using The Coiled Coil Polypeptide For Inhibition of *in vitro* Viral Activity

The efficacy of the coiled coil polypeptide (which is synthesized as described in the preceding section) in treating HIV infection is investigated by the determination of the inhibitory effect of different concentrations of the coiled coil polypeptide on the formation of viral induced syncytia formation and on infection by cell-free virus *in vitro*. These steps are described below.

1. Inhibition of Infected Cell-Induced Syncytium Formation By The Coiled Coil Polypeptide

Serial concentrations of coiled coil polypeptide O (e.g., between 1-50 $\mu\text{g/ml}$) are tested for blockade of the cell fusion process which is induced by HIV. The HIV-

HIV-1_{LAI} virus (Popovic *et al.* (1984) *Science* 224:497-5080) is prepared as follows. HIV-1_{LAI} virus is propagated in CEM cells cultured in RPMI 1640 containing 10% fetal bovine serum. Supernatant from the infected CEM cells is passed through a 0.2- μ m filter and the infectious titer is estimated in a microinfectivity assay using the AA5 cell line to support virus replication. For this purpose, 25 μ l of serially diluted virus is added to 75 μ l of AA5 cells at a concentration of 2×10^5 per ml in a 96-well microtiter plate. Each virus dilution is tested in triplicate. Cells are cultured for 8 days by addition of fresh medium every other day. On day 8 postinfection, supernatant samples are tested for virus replication as evidenced by RT activity. The 50% tissue culture infectious dose (TCID₅₀) is calculated. The titer of the HIV-1_{LAI} stock used for these investigations, as measured on the AA5 cell line, is expected to be 10^7 TCID₅₀/ml. The two primary isolates are obtained from peripheral blood mononuclear cells (PB-MCs) of two infected donors, one from Brazil (HIV-1_{Br3}) and the other from Trinidad (HIV-1_{QZ2775}), by cocultivation with phytohemagglutinin-stimulated normal donor PBMCs in RPMI 1640 containing interleukin 2. The infectious titers of the primary virus-stocks are estimated by titration on phytohemagglutinin-stimulated normal human PBMCs in a 96-well microtiter plate, again by using RT activity released to the supernatant as evidence of successful infection. The infectious titer of both these isolates is estimated to be $\approx 10^3$ TCID₁₀₀/ml.

The virus is ultracentrifuged to remove non-virus-associated peptides or soluble CD4 as follows. A 0.4-ml sample of treated virus is layered onto 0.15 ml of phosphate-buffered saline containing 5% sucrose in a Beckman SW50 minitube (total capacity about 0.6 ml) and centrifuged at 35,000 rpm for 60 min at 4°C. The supernatant is aspirated and the virus pellet is resuspended in RPMI 1640 containing 10% fetal bovine serum.

The prepared virus is used in the cell fusion assay as follows. MOLT-4 cells ($\approx 7 \times 10^4$) are incubated with CEM cells (10^4) chronically infected with HIV-1_{LAI} in 96-well plates (half-area cluster plates; Costar) in 100 μ l of culture medium. Peptide inhibitors are added in 10 μ l and the cell mixtures are incubated for 24 hr at 37°C. At that time, multinucleated giant cells are estimated by microscopic examination at x40

magnification, which allowed visualization of the entire well in a single field. Protection from viral induce cell-cell fusion indicates that the coiled coil polypeptide is useful for antiviral therapy.

2. Inhibition of Infection By Cell-Free Virus Using The Coiled Coil Polypeptide

The ability of the coiled coil polypeptide to inhibit infection by cell free virus is used as an *in vitro* assay of the polypeptide's efficacy in the prevention and treatment of HINV infection *in vivo*. A range of concentrations (0-100 µg/ml) of the coiled coil polypeptide is incubated in triplicate with virus (about 500 TCID₅₀) and cells in 96-well microtiter plates for 8 days. On the 8th day, supernatant is tested for reverse transcriptase (RT) activity as evidence of successful infection. A RT microassay is used. Supernatants from virus/cell cultures are made 1% (vol/vol) in Triton X-100. A 10-µl sample of supernatant is added to 50 µl of RT cocktail in a 96-well U-bottom microtiter plate and incubated at 37°C for 90 min. The cocktail contains 75 mM KCl, 2 mM dithiothreitol, 5 mM MgCl₂, poly(A) (5 µg/ml: Pharmacia cat. no. 27-4110-01), oligo(dT) (0.25 unit/ml; Pharmacia cat. no. 27-7858-01), 0.05% Nonidet P-40, 50 mM Tris-HCl (pH 7.8), 0.5 µM nonradioactive dTTP, and [α -³²P]dTTP (10µCi/ml; Amersham cat. no. PB.10167; 1 Ci = 37 GBq). After incubation, 40 µl of reaction mixture is applied to a Schleicher & Schuell NA45 membrane (or Whatman DE81 paper) saturated in 2x SSC (0.3 M NaCl/30 mM sodium citrate, pH7) in a Schleicher & Schuell Minifold over one sheet of GB003 filter paper. Each well of the minifold is washed four times with 200 µl of 2x SSC. The membrane is removed and washed twice in a Pyrex dish with an excess of 2x SSC. Finally the membrane is drained on absorbent paper, placed on Whatman no. 3 paper, covered with SaranWrap, and exposed to film overnight.

The reduction or absence of RT activity in sample treated with the coiled coil polypeptide as compared to controls which are not treated with the coiled coil polypeptide indicate that the coiled coil peptide which is specific for the HINV gp41 polypeptide is useful for antiviral therapy and/or prevention.

Advantages of the present invention

Traditional biological approaches to the study of disease are limited by the ability of existing nucleic acid-based probes to target only nucleic acids. While antibody-based probes partly overcome this problem in that they target polypeptide sequences, antibodies are both expensive and time-consuming to produce since they require purification of the target polypeptide and maintenance of animals. Moreover, functional antibodies cannot be expressed in intracellular compartments, and their use as biological probes often suffers from varying degrees of antigen expression, non-specific binding and adverse immunogenic reactions.

As is apparent from the data presented herein, the present invention solves the problems encountered in the prior art. The methods of the present invention provide the advantage that any polypeptide products which contains a coiled coil region, and which is involved in any disease state may be specifically and exclusively targeted to the exclusion of other polypeptides. The methods of the present invention provide simple and inexpensive methods for the design and production of polypeptide probes; probe sequence design is based on the simple selection of preferred amino acid pairs, and production is based on routine and automated chemical polypeptide synthesis or recombinant expression systems. Moreover, coiled-coil polypeptides in their native state may be easily targeted using the methods of this invention without the necessity for further purification or manipulations. Additionally, the methods of the present invention are sensitive in that they are capable of detecting physiologically relevant endogenous cellular concentrations of polypeptides.

Accordingly, it is to be understood that the drawings and descriptions in this disclosure are proffered to facilitate the comprehension of the invention and should not be construed to limit the scope thereof. Other improvements and modifications which become apparent to persons of ordinary skill in the art only after reading this disclosure, the drawings and the following claims are deemed within the spirit and scope of the present invention.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

C L A I M S

We claim:

1. A heterospecific polypeptide probe, comprising a substantially purified polypeptide comprising at least one probe coiled coil region capable of heterospecific oligomerization with a target coiled coil region, wherein the polypeptide sequence of said probe coiled coil region differs from the polypeptide sequence of said target coiled coil region with respect to at least one core residue.

2. A probe according to Claim 1, wherein the polypeptide sequence of said probe coiled coil region differs from the polypeptide sequence of said target coiled coil region with respect to at least one edge residue such that the charge on the edge residues in said probe coiled coil region is the same and is different from the charge on the edge residues in said target coiled coil region.

3. A probe according to Claim 1, wherein the hetero-oligomer between said probe coiled coil region and said target coiled coil region is more stable than the homo-oligomers formed by either said probe coiled coil region or said target coiled coil region based on measurement of melting temperature at a fixed total protein concentration or dissociation constant (Kd).

4. A hetero-oligomer formed between a heterospecific polypeptide probe according to any of Claims 1-3 and a target polypeptide.

5. A method for making a heterospecific polypeptide probe capable of heterospecific oligomerization with a target polypeptide, comprising:

providing a probe polypeptide having a probe coiled coil region corresponding to a target coiled coil region of said target polypeptide, wherein the polypeptide sequence of said probe coiled coil region differs from the polypeptide sequence of said target coiled coil region with respect to at least one core residue.

6. The method of Claim 5, wherein said probe polypeptide sequence is selected so as to optimize the packing and polarity of the core residues in said probe coiled coil region and in said target coiled coil region.

7. An isolated polynucleotide sequence encoding the polypeptide of
5 Claims 1 or 2.

8. A method for detecting a target polypeptide having a target coiled coil region, comprising:

10 contacting a sample suspected of containing said target polypeptide with a probe polypeptide having a probe coiled coil region capable of heterospecific oligomerization with said target coiled coil region of said target polypeptide; wherein the polypeptide sequence of said probe coiled coil region differs from the polypeptide sequence of said target coiled coil region with respect to at least one core residue; and

detecting the formation of said hetero-oligomer.

Figure 1

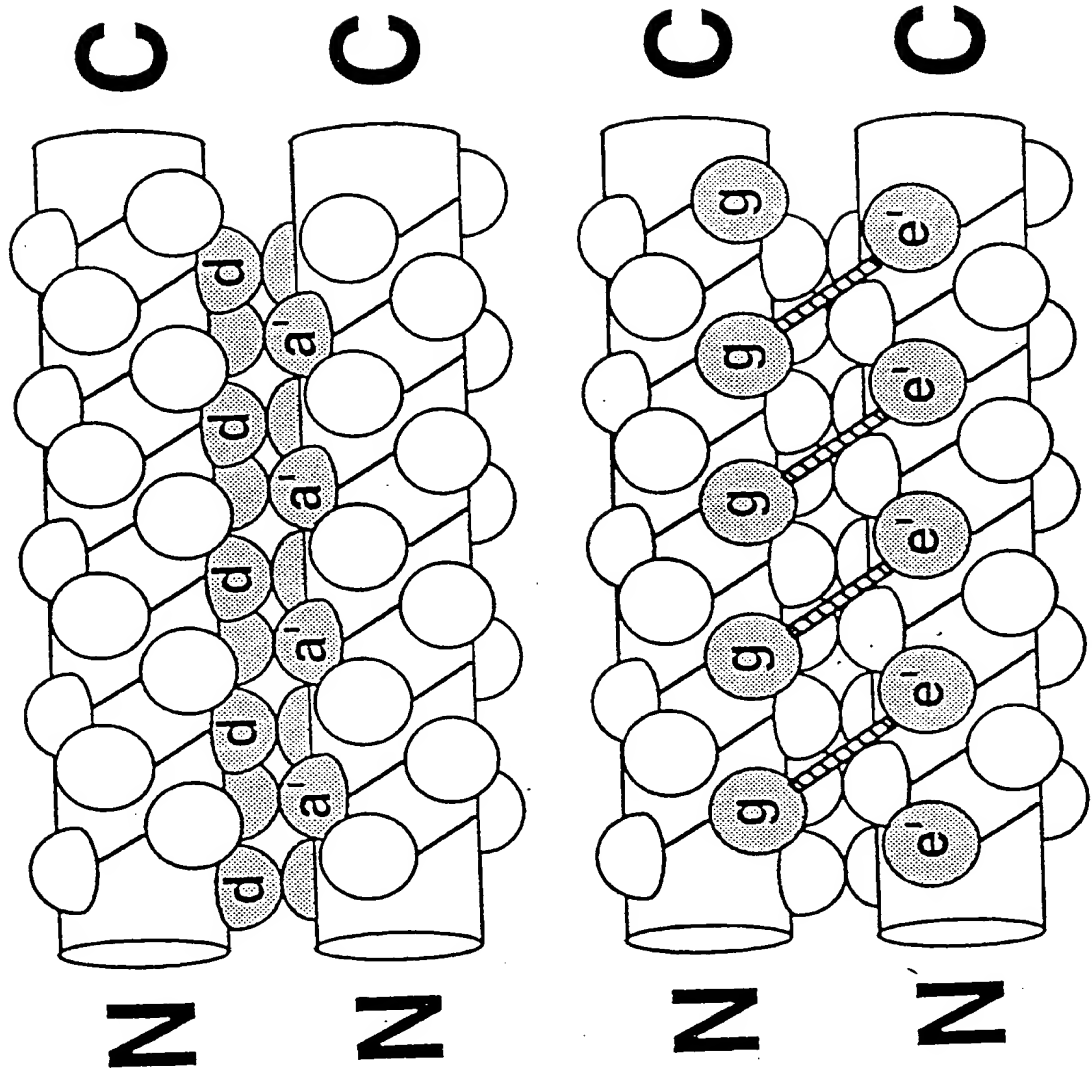


Figure 2

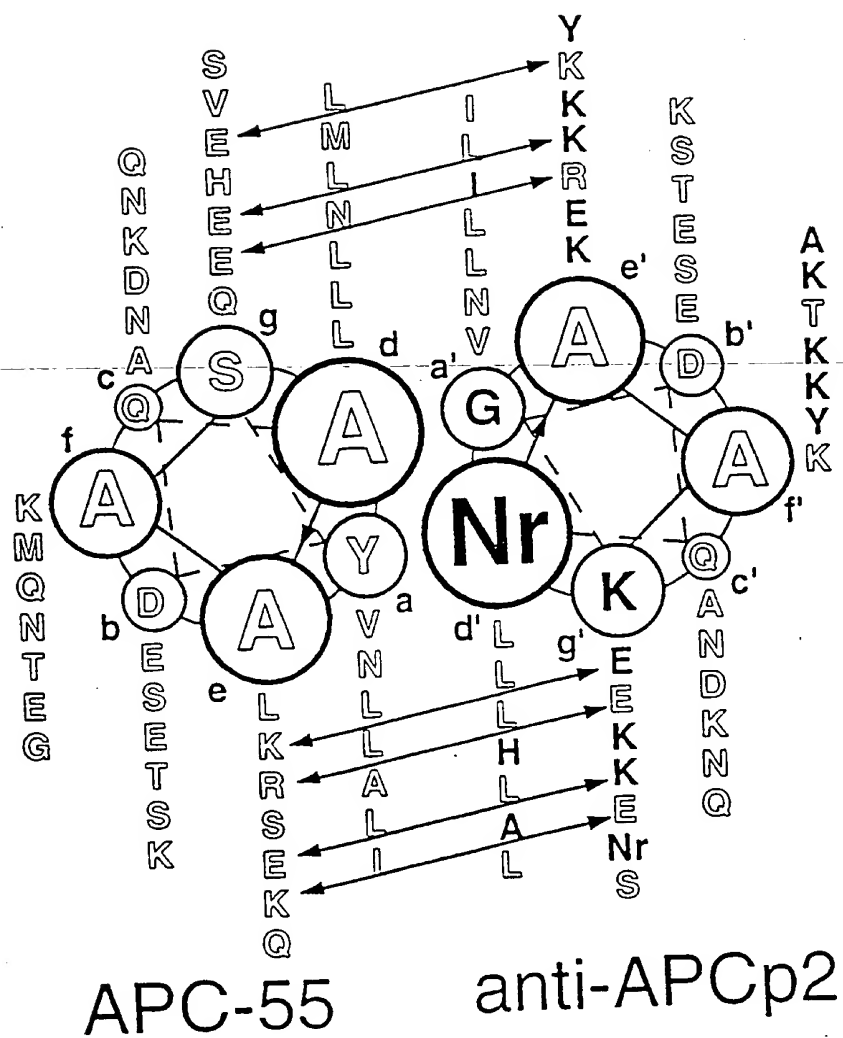


Figure 3

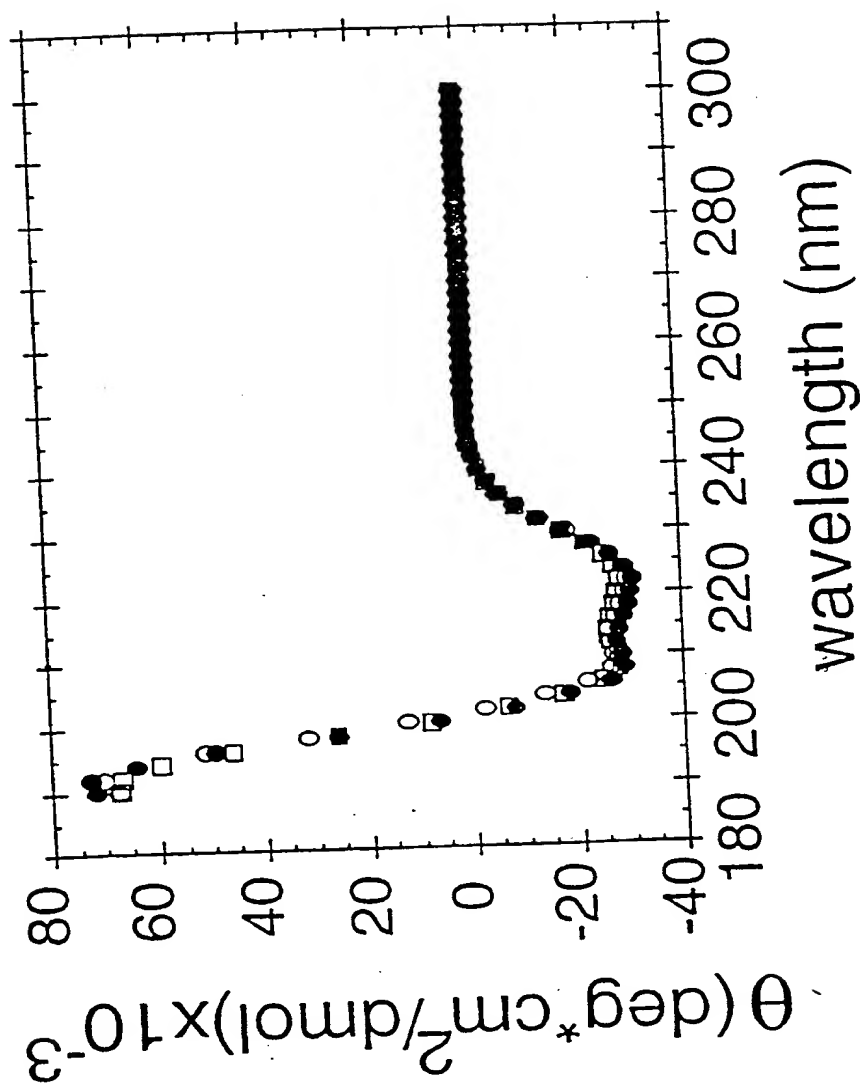


Figure 4

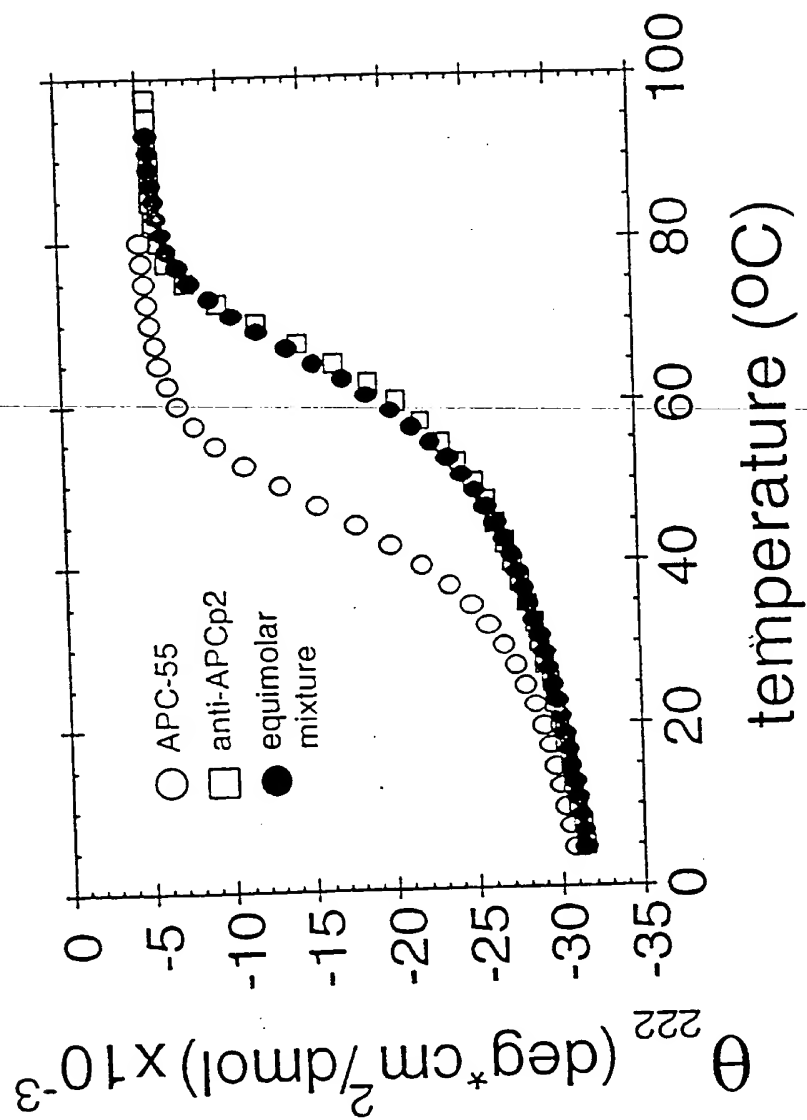


Figure 5

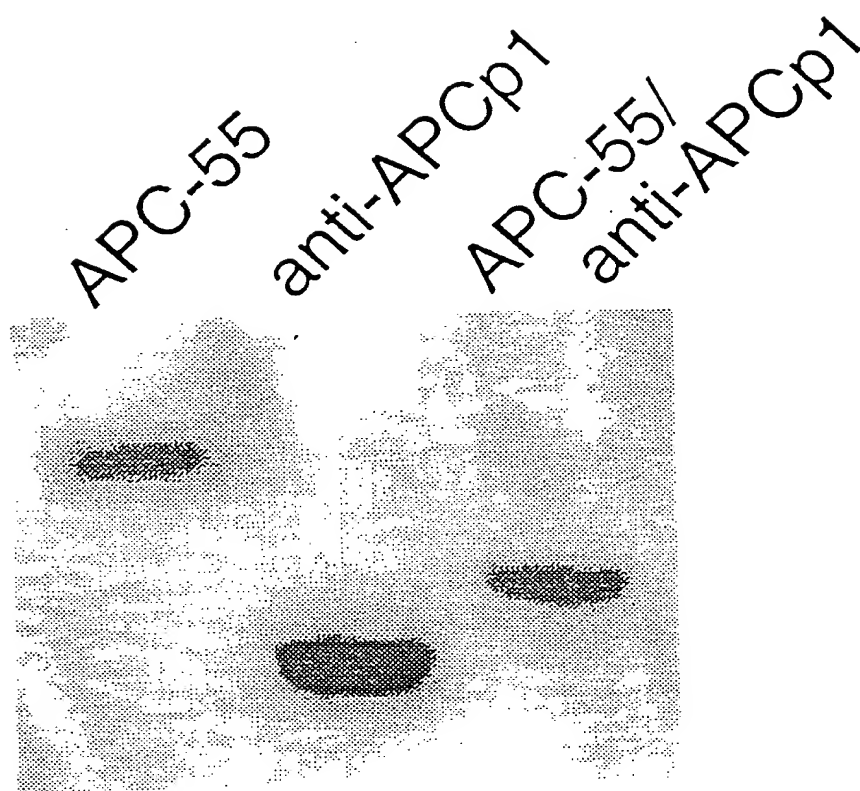
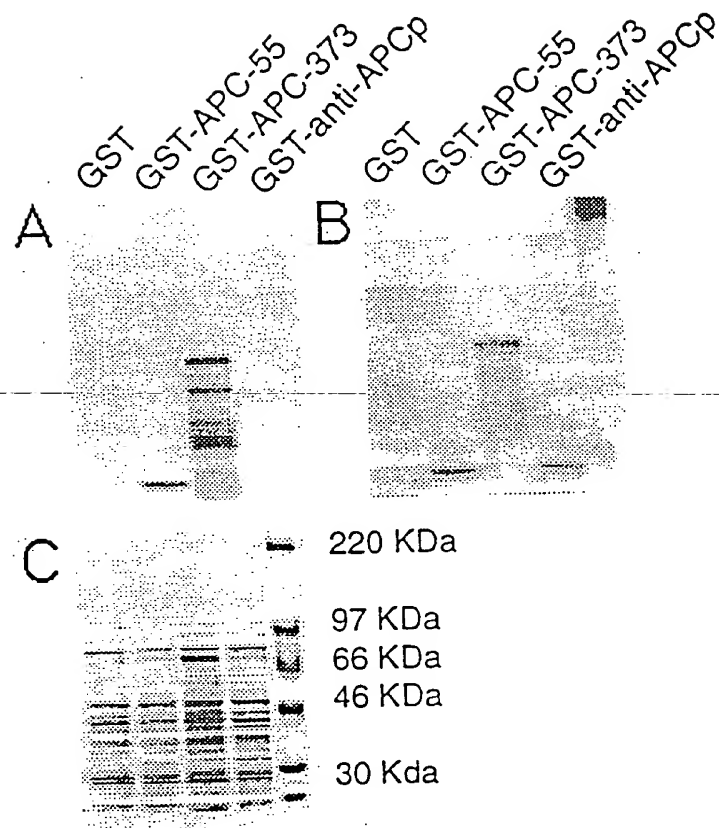


Figure 6



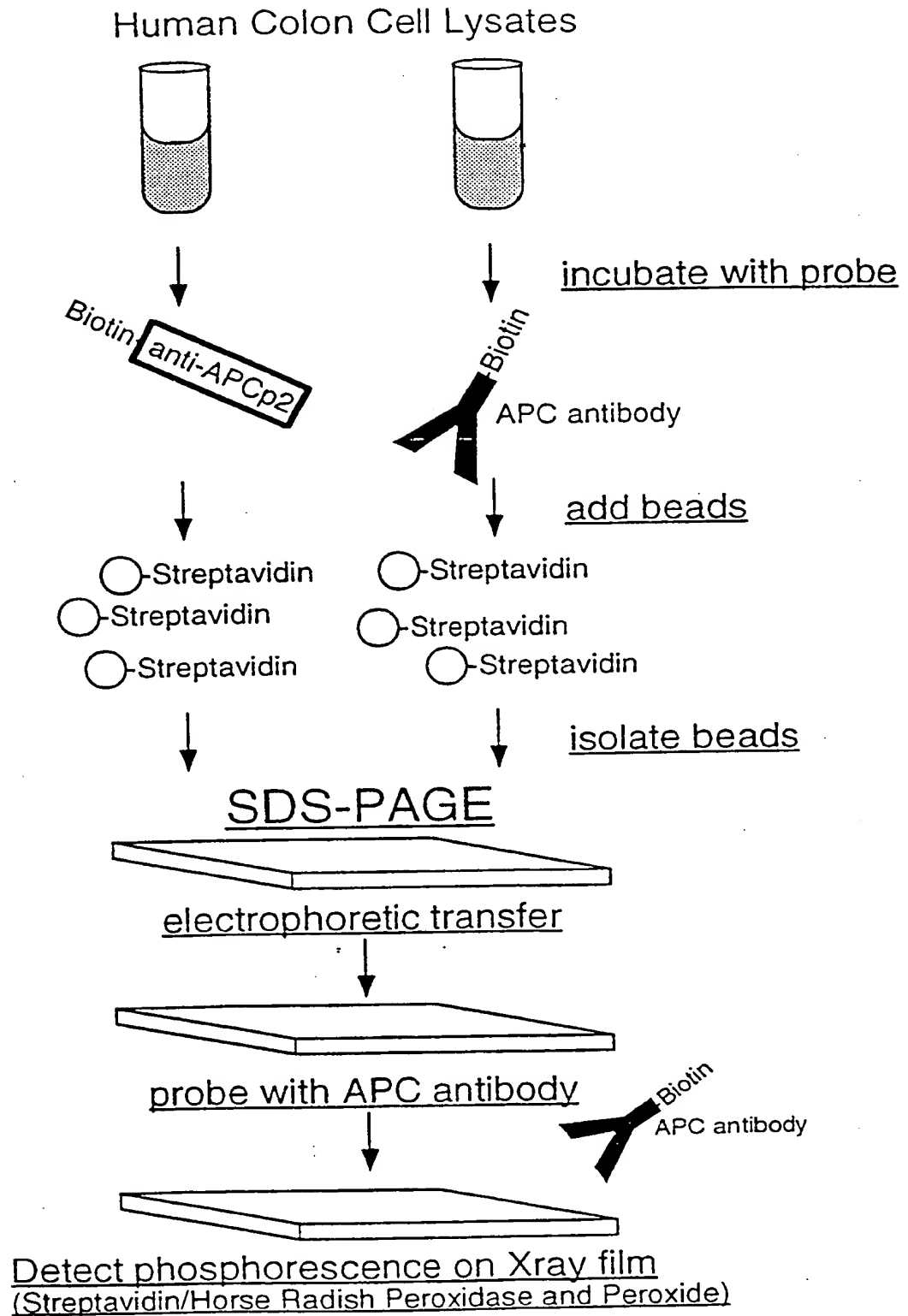
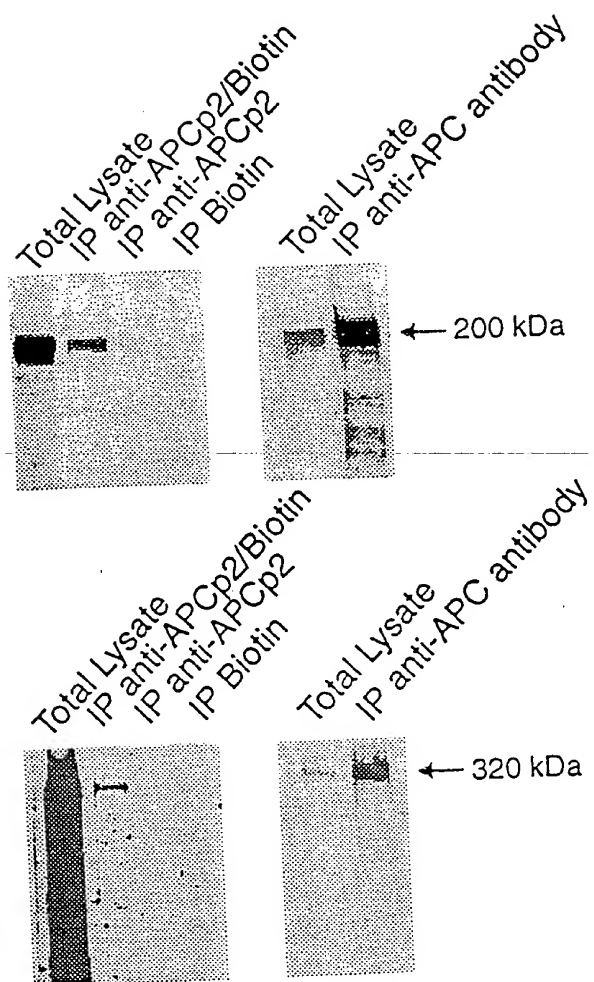
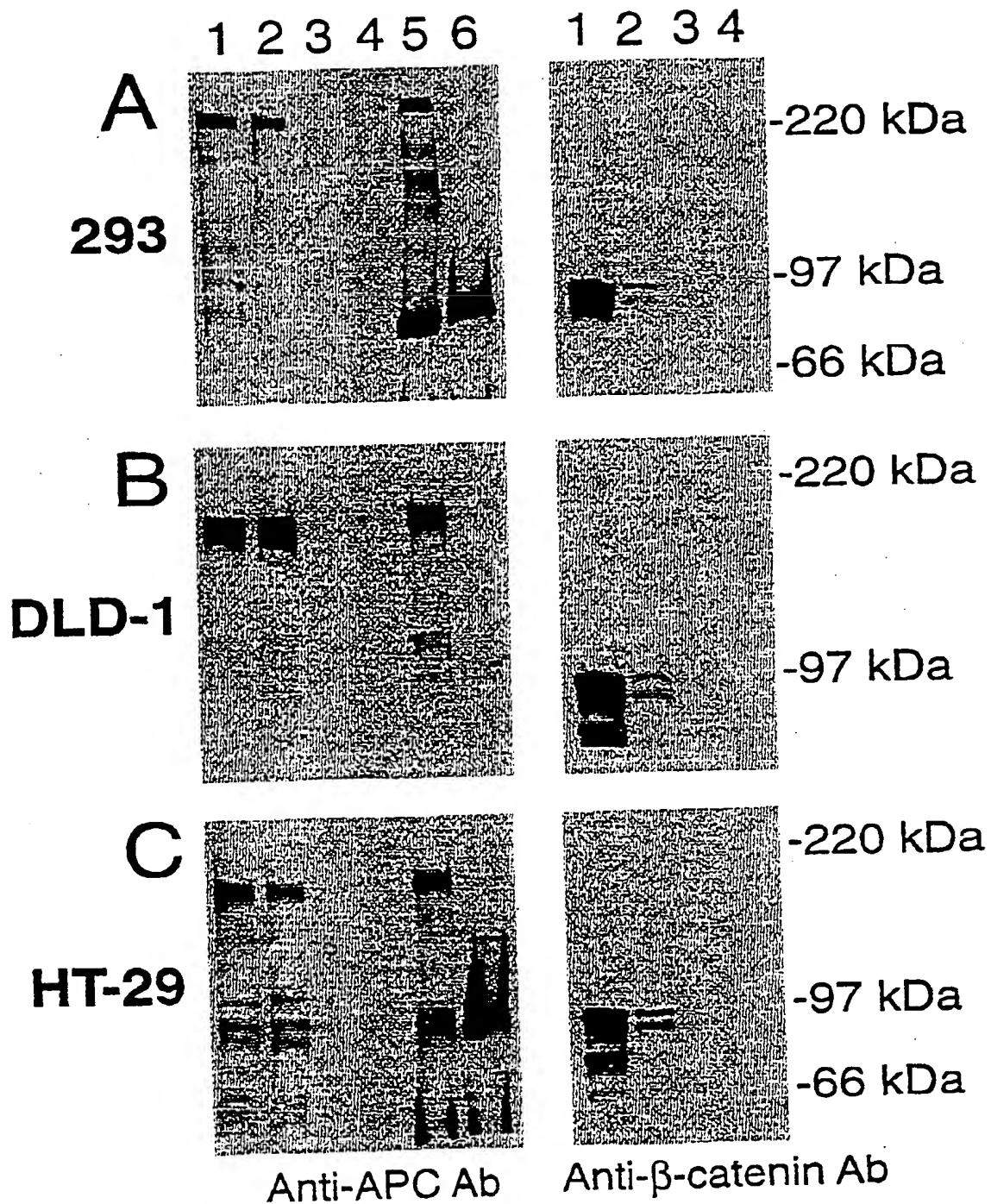
7/9
Figure 7

Figure 8



9/9

FIGURE 9



INTERNATIONAL SEARCH REPORT

Int. l. Application No

PCT/US 98/01871

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/11 C07K14/00 C07K14/47 C07K14/025 C07K14/39
C07K14/435 C07K14/16 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	WO 96 20953 A (UNIVERSITY OF NORTH CAROLINA) 11 July 1996 see page 2 - page 4 ---	1-8
X	WO 94 28920 A (DUKE UNIVERSITY) 22 December 1994 see page 25 - page 30 ---	1-8
X	WO 94 16109 A (WHITEHEAD INSTITUTE) 21 July 1994 see page 12 - page 20 ---	1-8
X	WO 94 02505 A (DUKE UNIVERSITY) 3 February 1994 see the whole document --- -/--	1-8

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

24 June 1998

Date of mailing of the international search report

09/07/1998

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INTERNATIONAL SEARCH REPORT

Int. l. Application No

PCT/US 98/01871

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation or document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>S NAUTIYAL ET AL.: "A designed heterotrimeric coiled coil" BIOCHEMISTRY., vol. 34, no. 37, 19 September 1995, EASTON, PA US, pages 11645-11651, XP002069185 see the whole document -----</p>	1-8

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/01871

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WO 9620953 A	11-07-1996	AU 4645396 A	24-07-1996
WO 9428920 A	22-12-1994	US 5464933 A	07-11-1995
		AU 7042694 A	03-01-1995
		EP 0774971 A	28-05-1997
		JP 8511525 T	03-12-1996
WO 9416109 A	21-07-1994	NONE	
WO 9402505 A	03-02-1994	AU 688733 B	19-03-1998
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		NZ 254640 A	24-04-1997
		US 5656480 A	12-08-1997

Form PCT/ISA/210 (patent family annex) (July 1992)

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